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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

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Barbiturates—Are They Narcotics?*

By SAMUEL W. GOLDSTEIN†

The distinction between narcotics and hypnotics is discussed. Differences in authoritative opinions concerning the inherent dangers of the barbiturates are indicated. Data based on hospital records to barbiturate habituation are presented and the need for legislation regulating barbiturate distribution is emphasized. The author concludes that legally barbiturates should not be classified as narcotics.

THE PROBLEM of adequate control of the distribution of barbiturates is recognized by many. The seriousness of the situation is shown by statistical data (1–3). The solution of the problem is being diligently sought by professional and lay groups. The medical and pharmaceutical groups seek to achieve control of barbiturate distribution by sponsoring effective uniform state laws. The lay groups seek to have the barbiturates legally classified as narcotics and made subject to all the regulations applying under the Federal Narcotic Act. Responsible public health officials are acutely aware of the barbiturate problem and agree that legislation of some kind is necessary. Dr. Paul Dunbar, U. S. Commissioner of Foods and Drugs, during a recent discussion of control of barbiturate distribution, stated: "A federal law would be too complicated and drastic. Uniform state laws would be more satisfactory" (4).

NARCOTICS AND HYPNOTICS

The barbiturates are generally referred to as hypnotics. What is the difference between narcotics and hypnotics? Medical dictionaries (5) list the two terms without cross-references. The definitions for "narcotic" state that stupor or sleep is produced and that pain is relieved. "Hypnotic" is defined as a drug that induces sleep. "True hypnotics" produce normal sleep. Other hypnotics include chloroform, opium, and other drugs that have an anodyne effect. Apparently the lexicographers interpret the two terms as having separate meanings. European medical writers customarily use the term "narcotic" to include hypnotics and frequent references are made to the barbiturate narcotics. American medical literature contains many statements about the hypnotic effect and the narcotic effect of

* Received Jan. 14, 1947, from the State of Maryland Department of Health, Baltimore.
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barbiturates but almost always refers to the barbiturates as hypnotics. Finally we have the legal definition of "narcotic drugs" as "coca leaves, opium, cannabis, and every substance not chemically distinguishable from them" (6).

Undoubtedly the main reason for passing the Federal Narcotic Act in 1914 was the rapid increase in the number of addictions to opiates and coca derivatives. During the period that this law has been in force the term "narcotic" has been accepted as applying to opiates, coca derivatives, and cannabis or marihuana. The question now being raised is this: Should the barbiturates be added to this group of drugs?

Among physicians there are honest differences of opinions as to the dangerous nature of barbiturate medication. Shelton (7) states ". . . . . the barbituric acid derivatives produce severe pathological changes in the organism when given in large doses over any moderate length of time. Even the small therapeutic doses when frequently repeated cause a moderate amount of tissue destruction. The barbiturates have an affinity for nervous tissue cells and destruction is most marked in the higher centers of the central nervous system." G. W. Robinson, Sr. (8) states, "In the treatment of mental ill health, barbiturates have a place, but only to meet emergencies and temporarily to relieve the patient of insomnia and mental distress. It is a form of medication which may be temporarily helpful in relieving symptoms but if continued over a period of days or weeks will do much more harm than good." Grogan (9) discusses the various uses of barbiturates in mental hospitals. He describes the results obtained, and continues: "All this is attained with no harm to the patient. I have never seen barbiturates, used in the ways described, bring about addiction to the drug. The few cases of barbiturate addiction that have come to my attention had an extremely apparent underlying psychiatric basis for their addiction." Grogan stresses the point that in all cases where barbiturates are administered over a long period of time, the minimum effective dose for the patient should be established and maintained.

Moore and Gray (10) in their discussion of patients who were believed to have a psychosis due to drugs (including barbiturates) or exogenous chemical poisons, state: "Many patients cleared mentally in a short time after withdrawal of the drugs. A few grew steadily worse, particularly those who had been using morphine and opium, probably due to the onset of withdrawal symptoms which are characteristically seen in users of the narcotic phenanthrene derivatives. . . Present knowledge concerning dependence on drugs is negative, for this phenomenon appears only upon withdrawal of drugs. The phenanthrene derivatives (morphine, etc.) appear to produce more dependence than do some of the coal tar drugs (barbiturates, for example). Persons who use drugs are motivated by the same mechanisms as obtain in alcoholism, and it is pointed out that drugs, other than alcohol, do not approach the importance of that substance as contributory factors in the production of mental disease."

BARBITURATE ADDICTION

A large proportion of the barbiturate addiction cases are associated with opiate addiction. Many of these will return to complete dependence upon opiates as soon as these drugs become available. The sad fact is that the opiates will become available in spite of the truly valiant work of our too small group of federal narcotic control agents. If some other countries were nearly as diligent as the United States in the curbing of traffic in opiates, cocaine, and cannabis, the already admirable results of the efforts of our enforcement agents would be even more spectacular. Nevertheless, during the period of 1940–1945, which included the war years when importation of opiates was practically eliminated, the addictions recorded in certain hospitals showed that the ratio of barbiturate addictions to total addiction cases did not increase.

While the hospital records of addictions give a picture that is far from complete, they are at present the most reliable data available. Furthermore, the comparison of hospital data from two different periods gives an indication of the frequency of occurrence

of addictions due to all drugs and of barbiturate habituation.

Hambourger (2) reported that for the decade 1928–1937 thirteen hospitals listed eighty-five cases under barbiturate addiction, an incidence of about one case for every 15,000 admissions. He reported more complete data for the following five hospitals: Baylor University, Dallas, Texas; Boston City, and Peter Bent Brigham, Boston; Presbyterian, New York; Michael Reese, Chicago. These hospitals had combined admissions for all causes of 761,923 cases. They had 398 cases of addiction to all drugs (excluding chronic alcoholism), forty-three of which, or 10.8 per cent. involved barbiturates; about one of every nine addiction cases.

The present author has compiled data for the six-year period 1940–1945, from the records of the following hospitals: Baylor University, Dallas, Texas; Boston City, Boston; Cleveland City, Cleveland; Presbyterian, New York; Presbyterian, Michael Reese, and University Clinics, Chicago; Walter Reed General, Washington, D. C.; St. Mary's, St. Louis; Baltimore City, Johns Hopkins, Mercy, Sinai, Union Memorial, and University, Baltimore. These hospitals had combined admissions for all causes of 1,138,887 cases. They had 715 cases of addiction to all drugs (excluding chronic alcoholism), sixty-eight of which, or 9.5 per cent, involved barbiturates; about one of every eleven addiction cases. The incidence of barbiturate addiction in the total admissions was one case in every 17,000 admissions. These data show that the frequency of barbiturate addiction compared to total admissions and to addictions to all drugs decreased in the 1940–1945 period. They also show that the frequency of addictions to all drugs compared to total admissions increased from one in every 1900 admissions during 1928–1937 to one in every 1600 admissions during 1940–1945.

This does not mean that the entire barbiturate situation improved during the years following 1937. Data published by Goldstein (1) show that this is far from true. But the present data emphasize the far lesser danger of addiction by barbiturates than by other habit-forming drugs, especially those legally classified as narcotics.

WITHDRAWAL SYMPTOMS

While continued daily use of barbiturates can lead to habituation, the almost complete absence of withdrawal reactions is in marked contrast to the reactions produced when drugs are withheld from opiate addicts. Barbiturates do not produce the euphoria or sense of well-being in the same sense or extent that is produced with opiates, cocaine, or cannabis. Barbiturates are not analgetic or pain-relieving, as are the opiates. The habituation developed on long usage of barbiturates is broken very easily compared with the addictions to opiates and cocaine. The barbiturates, when used alone, do not cause the psychic reactions which lead marihuana users to commit criminal acts. The continued use of opiates leads to the development of such a tolerance to the drug that addicts who desire euphoria are driven to the use of ever-increasing doses, sometimes ten or twenty times more than would be fatal to an unaccustomed individual (11). With barbiturates, a certain amount of physical adaptation is readily acquired, as with alcohol; but there is little real tolerance for large doses. Prolonged use of barbiturates by psychologically susceptible persons may cause the development of psychical degeneration and mania, as with alcohol, but these are often absent after long continued daily administration. With opiates, daily use will lead to addiction in ten days with susceptible individuals, and in twenty to twenty-five days in those with normal emotional stability (12).

The following case history, submitted by I. Kaplan, M.D. (13), is an example of the rational use of barbiturates over an extended period of time. A 60-year old male suffered an attack of coronary thrombosis. To insure complete rest, he was given 1 grain of phenobarbital sodium every four hours and $1^1/_2$ grains of pentobarbital sodium each night for a period of four weeks. Following this, the daily administration was limited to the nightly dose. The case was complicated by the development of an embolism during

the third week. After nine weeks of daily barbiturate administration, the improved patient showed no signs of drug intoxication, but showed sufficient drug dependence to demand his "sleeping pill" each night. During the tenth and eleventh weeks the patient, on his physician's advice, cut his sleeping pills down to one every other night. When he became ambulatory, during the twelfth week, the administration of barbiturates was discontinued with no apparent ill effect to the patient.

In 1940, G. W. Robinson, Jr., M.D. (14) discussed the seriousness of the barbiturate situation, and made the following statements: "It should not be necessary to wait for lawmakers [to correct the condition]. The physicians of the country are largely responsible for the situation. We have been too gullible in accepting the statements of the manufacturers and, worse if possible, we have passed those statements on to the laymen. Patients have been told by the family doctors, the consulting specialists, their nurses, and others in authority that they can take this medicine as long as they want to, that it will not hurt them and will not form a habit. There may have been a time when the knowledge of the dangers of these drugs was so limited that such statements could be made with sincerity but that day is past, ..."

Robinson may have been too harsh with his colleagues, but surely many physicians, pharmacists, and nurses still must be convinced of the dangers in barbiturate usage. The educational program should be pushed. The legislative program is being activated. The question as to how severe the legislative restrictions must be to achieve proper control has been studied and reported by Fischelis (15). The Legislative Committee of the AMERICAN PHARMACEUTICAL ASSOCIATION and the National Drug Trade Conference have endorsed a model Uniform State Barbiturate Act. The various state pharmaceutical and medical societies are studying this bill in order to make any necessary changes before its introduction to the state legislatures for enactment. This legislation should go far to correct the faults in the present system of barbiturate distribution.

The classification of barbiturates as narcotics may have some valid basis when considered only from a pharmacological viewpoint. But, taking the foregoing data into consideration, the answer to the question: "Should the barbiturates be classed as narcotics legally?" is "No."

SUMMARY

1. The ambiguous uses of the terms "narcotic" and "hypnotic" are discussed.

2. The differences in opinion among physicians regarding the dangerous nature of the barbiturates is indicated.

3. Hospital records for the periods 1928–1937 (I) and 1940–1945 (II) yielded the following data:

(*a*) Addictions to all drugs (chronic alcoholism excepted): Period I. One in every 1900 admissions. Period II. One in every 1600 admissions.

(*b*) Addictions to barbiturates: Period I. One in every 15,000 admissions. Period II. One in every 17,000 admissions.

(*c*) Relationship of barbiturate habituation to total addictions: Period I. One in every nine, or 10.8 per cent. Period II. One in every eleven, or 9.5 per cent.

4. The need for effective legislation to regulate barbiturate distribution is emphasized.

5. The question of legally classifying barbiturates as narcotics is answered in the negative.

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Hydrophilic Properties of Certain Ointment Base Constituents*

By ALFRED HALPERN† and LOUIS C. ZOPF‡

A series of saturated C_{10} to C_{18} fatty alcohols were studied for their effect on the hydrophilic properties of petrolatum. An optimum concentration at which each fatty alcohol exerts its maximum effect is noted. Cetyl alcohol has the lowest optimum value, but octadecyl alcohol (stearyl alcohol), having a slightly higher optimum value, exerts the strongest effect on the hydrophilic properties of petrolatum. The values obtained by two methods are compared and the practical value of this property of the fatty alcohols is discussed. A comparative study of these alcohols in bases analogous to that described in the Swiss Pharmacopoeia V is made.

A METHOD for the extemporaneous formulation of a hydrophilic ointment base is of decided advantage to the pharmacist and physician. The need frequently arises for a base that will incorporate a definite quantity of water or a solution of a medicament. While many excellent bases that will take up varying amounts of water have been reported, these bases, for the most part, are fixed in their water content. There is very little flexibility in their hydrophilic properties. We realized the possibilities toward improved ointment therapy through the development of a system of formulation, whereby the amount of water taken up by an ointment base can be predicted and how such a base might be altered to accommodate varying amounts of water without qualitatively changing the product. A knowledge of the effects of varying concentrations of ointment base constituents on the hydrophilic properties of the base as a whole would result in such a system. Thus, by quantitatively altering these agents, a base could be constructed to accommodate a predetermined amount of water or solution of a medicament.

This study was undertaken to extend the work of Casparis and Meyer (1) and to investigate the hydrophilic properties of a homologous series of saturated fatty alcohols. We investigated the saturated fatty alcohols from C_{10} to C_{18}. The "water numbers" of the individual alcohols were noted (Table IV) and a study of the combined effects of the fatty alcohol and wool fat on the water-absorbing properties of petrolatum was also carried out.

We wish to emphasize that the resulting preparations of the fatty alcohols and petrolatums are not to be construed as a recommended ointment base, nor do we propose that the hydrated mixture be used in such a manner, without further adjustment. It is noted, however, that the Swiss Pharmacopœia V has official a base that is analogous to the fatty alcohol-wool fat-petrolatum group (Part II) (5).

PART I

In 1935 Casparis and Meyer published their results of a study of the hydrophilic properties of certain ointments and ointment bases (1). They described a method for determining the "water number" of a fat or ointment base. Of particular interest is their statement that the water number of a mixture is roughly the sum of the increases produced by the individual components. Among the substances investigated, cetyl alcohol was found to increase the water number of petrolatum three to four times. An optimum concentration of the cetyl alcohol in petrolatum was noted by the investigators. At this optimum, cetyl alcohol causes its maximum potentiation of the water-absorbing properties of petrolatum.

The "water number" as defined by Casparis and Meyer, is the largest amount of water which 100 Gm. of an ointment base or fat will hold at normal temperature (20°). Their method of determining the water number is as follows:

Method 1—"The incorporation of the maximum amount of water was effected in a mortar by adding water to the melted base and triturating until cool. If no water remained, more was added in small amounts until no more was taken up. The ointment was transferred to a jar and kept in a refrigerator for several hours at about 0°. It was then al-

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lowed to come up to room temperature and rubbed on a slab with a spatula. This procedure was repeated until no more water exuded. The water present in a 5- or 10-Gm. sample of the base was determined by the method of Pritzker and referred to 100 Gm. of base."(2).

The water number is calculated in the following manner:

Weighed amount of saturated base	$= x$
Water content	$= y$
Weight of base alone	$= (x - y)$
Water number	$= \frac{100y}{(y - x)}$

The water numbers of various concentrations of a homologous series of fatty alcohols (Table I) in petrolatum were determined by this method (Table II). The study was limited to concentrations up to 15% of the fatty alcohol in petrolatum because our objective was to learn the effects of the fatty alcohols at minimal concentrations. We were not concerned with any further physical property that is contributed by the fatty alcohols to the base at higher concentrations.

TABLE I.—FATTY ALCOHOLS TESTED

Fatty Alcohol	Formula	Grade[a]
Decanol	$C_{10}H_{21}OH$	Technical
Lauryl alcohol	$C_{12}H_{25}OH$	Technical
Myristyl alcohol	$C_{14}H_{29}OH$	Unknown
Cetyl alcohol	$C_{16}H_{33}OH$	Cosmetic
Stearyl alcohol (Stenol)	$C_{18}H_{37}OH$	Cosmetic
Petrolatum (yellow)		U. S. P.
Petrolatum (white)		U. S. P.
Wool fat		U. S. P.

[a] In no case were the physical properties observed of the fatty alcohols those given in the Handbook of Chemistry and Physics.

another method was employed to determine the water numbers of the various petrolatum fatty alcohol mixtures.

Method 2.—A 10-Gm. portion of the appropriate petrolatum-fatty-alcohol mixture was triturated in a mortar with water delivered from a burette 0.5 cc. at a time. When no more water was taken up, the mixture was chilled for an hour in an ice bath. It was then allowed to warm to room temperature and was levigated on a slab. If water was exuded by levigation, the process was repeated on a fresh sample, using 0.1 cc. less of water. This was repeated until a level was reached at which no water could be exuded by levigation. As a further check, the amount of water taken up by 10 Gm. of the mixture was referred to 100 Gm. and this value checked by adding this quantity of water to 100 Gm. of the mixture and treating as above. The values recorded are those for 100 Gm. of the mixture (Table III).

DISCUSSION

The values obtained indicate that there is a definite relationship between the concentration of the fatty alcohol and the amount of water held by the mixture. It is important to note that the water number is specific for the fat. Thus Casparis and Meyer report (1-3) that while 4% of cetyl alcohol in petrolatum raised the water number of petrolatum from (9.3-15.6) to (38.8-51.5), a 3% of cetyl alcohol in hydrogenated peanut oil raised the water number of the peanut oil from 75.4 to 185.7. Lard had its water number raised from 7.5 to 244.9 by a 3% concentration of cetyl alcohol. For this reason, the fat phase should always be indicated in any discussion of the water number of a substance.

TABLE II.—WATER NUMBERS OF FATTY ALCOHOL-PETROLATUM MIXTURES DETERMINED BY METHOD 1

	Concentration in White Petrolatum											
Fatty alcohol	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%	12.5%	15%
Decanol	10.14	11.2	12.75	13.26	14.61	15.43	16.15	16.8	17.2	17.8	18.1	16.2
Lauryl alcohol	14.1	17.2	18.3	19.4	21.0	22.7	23.5	24.6	25.4	23.2	21.4	19.2
Myristyl alcohol	21.7	22.8	26.5	28.7	31.2	30.8	32.5	34.8	30.3	31.4	28.0	24.1
Cetyl alcohol	31.1	36.2	38.0	38.5	36.1	36.4	35.1	34.0	33.2	31.2	30.6	29.5
Stearyl alcohol	32.0	35.1	39.3	41.2	42.5	43.2	40.1	40.5	39.7	38.0	35.6	33.2
	Concentration in Yellow Petrolatum											
Fatty alcohols	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%	12.5%	15%
Decanol	10.7	12.1	12.9	13.5	14.1	15.6	16.7	17.1	17.9	18.8	19.8	16.9
Lauryl alcohol	14.2	16.1	17.3	19.5	20.6	22.3	24.0	25.8	27.1	25.2	22.1	19.3
Myristyl alcohol	22.6	24.5	27.1	28.5	30.8	31.3	33.5	34.6	32.1	30.2	27.4	25.1
Cetyl alcohol	32.6	35.1	38.2	40.2	38.8	37.1	36.0	34.1	32.6	31.1	29.8	29.1
Stearyl alcohol	35.1	38.2	40.1	42.6	44.3	45.2	41.2	40.3	39.0	38.2	36.0	34.0
Water number of White petrolatum				9.0								
Water number of Yellow petrolatum				10.2								

The optimum concentration of the fatty alcohol in the petrolatum derived by this method was noted in Table IV.

As the authors (Casparis and Meyer) make the statement that this method does not have the exactness of other analytical methods since various interfering factors cannot be well controlled (1),

The purity of the fatty alcohol and the petrolatum is important in the application of the water number to extemporaneous formulation. A range of values has been obtained depending on the quality of the fatty alcohol and the petrolatum used. In order to standardize our results and eliminate as much as possible this source of variation, we used

TABLE III.—WATER NUMBERS OF FATTY ALCOHOL AND PETROLATUM MIXTURES DETERMINED BY METHOD 2

	Concentration in White Petrolatum											
Fatty alcohol	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%	12.5%	15%
Decanol, $C_{10}H_{21}OH$	11.3	12.1	13.5	14.2	15.1	15.1	15.7	16.1	17.0	17.2	17.8	15.1
Lauryl alcohol, $C_{12}H_{25}OH$	15.2	18.3	18.5	19.7	21.8	22.5	23.0	24.1	25.2	22.7	20.3	19.0
Myristyl alcohol, $C_{14}H_{29}OH$	21.0	23.2	26.1	29.0	30.1	30.3	31.3	32.8	30.0	28.1	26.0	23.2
Cetyl alcohol, $C_{16}H_{33}OH$	30.0	35.5	37.8	38.2	38.0	37.6	37.0	36.1	36.3	35.2	32.1	32.4
Stearyl alcohol, $C_{18}H_{37}OH$	33.1	36.5	38.2	39.0	40.7	42.3	42.0	41.3	40.2	38.7	37.5	36.8
	Concentration in Yellow Petrolatum											
Fatty alcohol	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%	12.5%	15%
Decanol, $C_{10}H_{21}OH$	11.2	12.7	13.8	14.8	16.0	16.3	17.1	17.8	18.0	18.4	19.1	17.8
Lauryl alcohol, $C_{12}H_{25}OH$	15.5	17.8	18.7	19.5	21.6	22.8	23.5	24.8	26.0	25.1	24.2	23.0
Myristyl alcohol, $C_{14}H_{29}OH$	20.0	23.4	25.6	28.2	30.3	30.8	32.0	33.2	31.2	29.2	27.1	24.8
Cetyl alcohol, $C_{16}H_{33}OH$	31.5	36.1	41.2	43.5	40.1	39.8	38.1	36.5	36.0	35.0	34.1	33.8
Stearyl alcohol, $C_{18}H_{37}OH$	35.0	38.2	39.3	41.2	43.1	45.3	43.0	41.6	42.0	40.0	38.2	37.1
Water number of white petrolatum			9.5									
Water number of yellow petrolatum			10.8									

a petrolatum described as meeting U. S. P. XII specifications and fatty alcohol commonly sold as the dermatological grade. However, it is important to note that in some cases these were designated as "technical grade." The purities of the fatty alcohols used are described in Table I.

TABLE IV.—OPTIMUM CONCENTRATION AND WATER NUMBERS OF FATTY ALCOHOLS

	Method 1		Method 2	
Fatty Alcohol	Optimum Concentration, %	Water Number	Optimum Concentration, %	Water Number
White Petrolatum				
Decanol	12.5	18.1	12.5	17.8
Lauryl alcohol	9.0	25.4	9.0	24.1
Myristyl alcohol	8.0	34.8	8.0	32.8
Cetyl alcohol	4.0	38.5	4.0	38.2
Stearyl alcohol	6.0	43.2	6.0	42.3
Yellow Petrolatum				
Decanol	12.5	19.8	12.5	19.1
Lauryl alcohol	9.0	27.1	9.0	26.0
Myristyl alcohol	8.0	34.6	8.0	33.2
Cetyl alcohol	4.0	40.2	4.0	43.5
Stearyl alcohol	6.0	45.2	6.0	45.3

Of the series studied, cetyl alcohol had the lowest optimum concentration and stearyl alcohol caused the greatest potentiation of the water number of petrolatum. The water number of petrolatum apparently increased with the increase in the length of the carbon chain. The concentration necessary to cause the maximum increase has its minima at a carbon chain length of 16. Thus, the fatty alcohols tested on both sides of cetyl alcohol require a greater concentration to exert their maximum effect on the water number of petrolatum. We hesitate to draw conclusions from a minimal amount of data; therefore we merely report these observations as properties of the fatty alcohols tested and not as a generalization on the fatty alcohols of the type $C_nH_{(2n+1)}OH$. We are continuing the investigation of the relationship of the lipophilic group to the hydrophilic group with regard to the effect on the water number of ointment base constituents.

Of the alcohols studied, decanol and lauryl alcohol possess a characteristic odor which detracts from their use in ointments. Myristyl, cetyl, and stearyl alcohols do not have this objectionable feature. All emulsions resulting from this study were stable "water-in-oil" types. "Sweating" was evidenced in the saturated mixtures kept at 45° overnight. However, preparations containing 3% to 5% less water than that indicated by the water number showed no "sweating" at this temperature. No "sweating" was observed with the saturated mixtures kept at room temperature (25°). This would seem to indicate that the water number is influenced by temperature.

PART II

One of the purposes of this study was to develop a means of extemporaneous formulation of hydrophylic ointment bases. Of particular interest in this regard is the statement that "the water number of a mixture is roughly the sum of the water numbers of its components" (2).

We decided to study the effects of a mixture of two fatty alcohols and petrolatum and wool fat-fatty alcohol-petrolatum on the water number of petrolatum. The methods used to determine the water number of these mixtures were those described in Part I of this paper. The fatty alcohols were used at their optimum concentrations (A) and the wool fat-fatty alcohol-petrolatum series was patterned after that described in the Swiss Pharmacopœia V (B). The type of formulas used were:

(A) Fatty alcohol I.....Optimum concentration
Fatty alcohol II....Optimum concentration
Petrolatum *q. s.*.....100 Gm.

(B) Wool fat.....10 Gm.
Fatty alcohol.....Optimum concentration
Petrolatum *q. s.*....100 Gm.

Casparis and Meyer investigated the Unguentum Cetylicum of the Swiss Pharmacopœia V and found

that it had a water number of 81.1 to 108.3. This base contains the optimum concentration of cetyl alcohol (4%) in wool fat (10%) and petrolatum (86%). The range of the water number was attributed to the type of petrolatum used.

Professor Rupp (4), studying a base containing myristyl alcohol in the place of cetyl alcohol in the above formula (B), found that it took up about 10% less water than the cetyl alcohol preparation. He further states that myristyl alcohol gives ointments which are inferior to cetyl alcohol as regards the taking up of water.

We repeated these determinations merely to complete our series. It was found that introducing the maximum amount of water (i.e., the amount indicated by the water number) produced a preparation that did not possess all of the physical properties desired in an ointment base. A preparation that was extremely soft resulted, particularly so in the mixtures containing over 110% of water, based on the quantity of fat phase. By using 10% to 15% less water than is indicated by the water number a preparation having an increased pharmaceutical elegance resulted.

The water number of a mixture of fatty alcohols and petrolatum (Formula A) is not the sum of the individual water numbers. There was some potentiation but this was not constant. In the cases where decanol and lauryl alcohol were used, a slight decrease in the water number was noted. The mixture of myristyl-cetyl, myristyl-stearyl, and cetyl-stearyl alcohols showed some potentiation but not that equivalent to the sum of their individual water numbers (Table V).

TABLE V.—WATER NUMBERS OF MIXTURES OF FATTY ALCOHOLS

Fatty Alcohols	White Petrolatum Calcd.	White Petrolatum Found	Yellow Petrolatum Calcd.	Yellow Petrolatum Found
Decanol + Lauryl	43.5	20.1	46.9	24.1
+ Myristyl	52.9	27.2	54.4	26.3
+ Cetyl	56.6	30.6	60.0	31.9
+ Stearyl	61.3	35.4	65.8	38.0
Lauryl + Myristyl	60.2	20.1	61.7	22.1
+ Cetyl	64.9	35.4	67.3	38.1
+ Stearyl	68.6	38.8	72.3	40.2
Myristyl + Cetyl	73.3	47.2	74.8	48.8
+ Stearyl	78.0	51.0	79.8	54.5
Cetyl + Stearyl	81.7	63.5	85.4	60.1

The water number of the formulas containing wool fat and a single fatty alcohol-petrolatum (Formula B) was roughly that of the sum of their individual components (Table VI).

The emulsions were all of the water-in-oil type.

This work is to be extended to include the polyhydroxy alcohols and the alcohol derivatives. We intend also to investigate the activity of the alcohols in other fats. The results of these investigations will be reported at a later date.

TABLE VI.—WATER NUMBER OF WOOL FAT, FATTY-ALCOHOL, AND PETROLATUM MIXTURES

	F. A., %	W. F., %	Pet., %	Yellow Petrolatum Calcd.	Yellow Petrolatum Found	White Petrolatum Calcd.	White Petrolatum Found
Decanol[a]	12.5	5[b]	82.5	96.6	64.1	98.3	62.1
Lauryl[a]	9.0	5[b]	87.0	103.9	82.5	105.6	86.3
Myristyl[c]	8.0	10	82.0	113.3	97.1	113.1	93.2
Cetyl[c]	4.0	10	86.0	117.0	108.3	118.7	104.1
Stearyl alcohol[c]	6.0	10	84.0	121.7	114.5	123.7	118.2

[a] An extremely soft preparation resulted. More water may have been taken up but it could not be levigated. It resembled a cream in consistency. The odor of the preparation was objectionable.
[b] A 5% concentration of wool fat in petrolatum has the same water number as a 10% concentration.
[c] A softened preparation resulted by the incorporation of the maximum amount of water to the base. Using 10% less water resulted in a stiffer base.

CONCLUSIONS

1. Of the series studied, cetyl alcohol had the lowest optimum concentration and stearyl alcohol caused the greatest potentiation of the water number of petrolatum.

2. "Sweating" was evidenced in the saturated mixtures and not in the unsaturated mixtures at higher temperature (45°). The results seem to indicate that the water number is influenced by temperature.

3. The water number of a mixture of fatty alcohols is not the sum of the individual water numbers.

4. The water number of a mixture of wool fat-fatty alcohol and petrolatum is "roughly" the sum of the water numbers of the individual components.

5. The emulsions were all of the water-in-oil type.

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Effect of Sodium 5-Allyl-5(Methylbutyl)barbiturate (Sodium Seconal) on Oxygen Consumption in Rats*

By NILKANTH M. PHATAK and EDWARD SAXEY

The results of a study of the effect of subanesthetic doses of Seconal on oxygen consumption are reported. Peoples' photographic method was modified to measure continuous oxygen consumption of rats. In 30 rats the average rate of oxygen consumption under control conditions was 4.5 cc. per minute which was reduced by Seconal to 4.0 cc. per minute with no change in respiratory rate. In 5 rats, not included in the results above, slight excitation was produced so that the oxygen consumption increased from 4.4 cc. per minute to 5.1 cc. per minute without effect on the respiration. These results show that in the majority of animals a significant reduction in oxygen consumption (metabolic rate) occurs with subanesthetic doses of Seconal without change in respiratory rate, or due to the effects of muscular relaxation.

GUEDEL (1) states that the "reflex irritability" of an individual under general anesthesia parallels the metabolic rate, which in turn reflects the oxygen demand of the body cells. Since preanesthetic sedatives contribute an appreciable share of the depression produced in anesthesia, the extent of their effect on oxygen consumption is of importance. The barbiturates may influence oxygen consumption through one or through a combination of the two following mechanisms: (*a*) direct (systemic) depression of intracellular enzymic oxidative processes, or (*b*) indirect cellular anoxia produced subsequent to respiratory center depression. The danger of anoxia is greater when the particular preanesthetic depressant and the size of its dose tend to depress the process of respiration more than the sum-total of vital, intracellular oxygen consumption. A desirable barbiturate thus is one which can afford a wide range, or safety margin, between the dose producing a definite decrease in oxygen consumption rate and that depressing the respiratory function.

Previous studies concerning effects of barbiturates on metabolic rate show contradictory results. Anderson, Chen, and Leake (2) in obtaining evidence for Guedel's metabolic gradient concept, found that Amytal, in therapeutic doses, produced a slight increase in the oxygen consumption rate of normal individuals. This effect, however, was reversed by larger doses. They also noted an increase in the respiratory rate after amytal administration.

Lee (3) injected adult rats subcutaneously and intraperitoneally with gradually increasing doses of Amytal and observed a corresponding decrease in the basal metabolic rate. The route of administration had no influence in his results.

Siebert and Thurston (4) studied effects of subcutaneous injections of Amytal in guinea pigs. They found a decreased rate of heat production in these animals. Kleiber and Saunders (5) injected rats subcutaneously and intraperitoneally with Amytal. They noted that thirty minutes after the intraperitoneal injections, the oxygen consumption rate decreased 68 per cent of that for the controls; and 80 per cent of that for the controls after subcutaneous administration. When the Amytal dose was adequate to produce anesthesia, a considerable decrease in the metabolic rate of the animals was noted.

The effects of barbiturates on metabolism are considerable when given in large doses, but in ordinary doses their action on metabolism is held to be negligible. As Peoples (6) puts it, "the effects of barbiturates on oxygen consumption are no more than would be expected from a similar degree of relaxation in sleep." Other experimental observations of Deuel on dogs (7) and of Rowntree (8) on human subjects also indicate the rather inconclusive effects of barbiturates on oxygen consumption.

Our study is limited to the effects of sub-

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cutaneous administration of subanesthetic doses of Sodium Seconal on the oxygen consumption of rats. This drug is metabolized predominantly through the liver. Since central nervous system depressant drugs produce rather pronounced metabolic effects through sympathetic stimulation (reflex adrenin mediation), we thought it would be of interest to note the effects of this type of short-acting barbiturate on the oxygen requirements of the body.

METHODS

A modification of Peoples' photomotographic method (9) was utilized to obtain visual records of a continuous oxygen consumption rate of young rats of both sexes. All animals served as their own controls since previous to Seconal injections, a record of the animal's oxygen consumption was obtained over a control period of sixty to ninety minutes. During this period the rats were accustomed to the closed oxygen system of the apparatus. By extending the control observations to longer periods it was possible to discount for the periodic contributions to increased oxygen consumption caused by the sporadic activity of the rats. (See Fig. 2.) Summating the results of these prolonged control and observation periods made it possible to obtain reliable "average" rates of oxygen consumption for comparison. (See Figs. 3 and 4.) The glass jar, in which the animal was placed, was immersed in a constant temperature water bath at 28° since the flow meter on the apparatus was calibrated at this arbitrary point. (See Fig. 1.) The temperature of the bath was regulated so as to not vary 0.2°. This procedure eliminated effects of variations of temperature entering into the calculations of the graphic oxygen consumption figures.

RESULTS

The results of observations on 30 rats injected subcutaneously with Sodium Seconal and accompanying data are presented in Table I. For comparison of dosage variation the results are also summarized in Table II.

It is apparent from the results that subanesthetic doses of Sodium Seconal, like other barbiturates, produce variable degrees of central nervous system depression depending on individual variation. Often the same dose produces increase of muscular activity

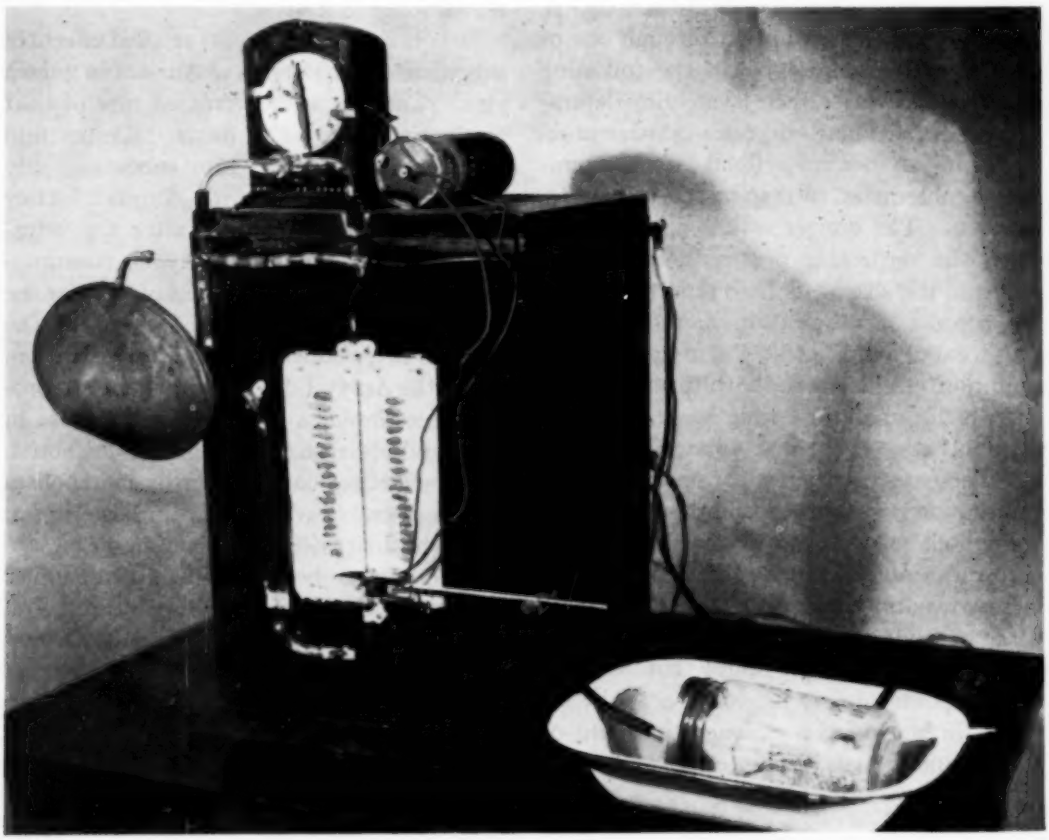

Fig. 1.—Metabolism apparatus. Modified Peoples' photographic metabolar.

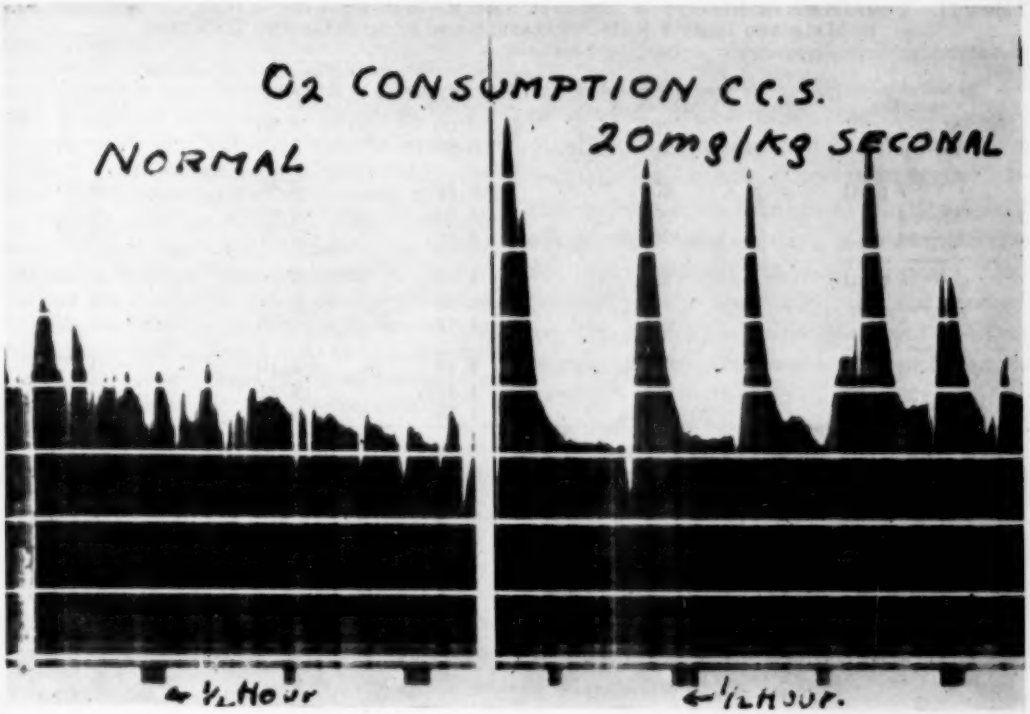

Fig. 2.—Showing periodic spurts of increased activity (sharp peaks on the right half of the record) of rats during the experimental period.

TABLE I.—EFFECT OF "SECONAL SODIUM" INJECTIONS ON O_2 CONSUMPTION RATE IN THIRTY WHITE RATS

Sex	Wt., Gm.	Dose, Mg./Kg.	Pre-Injection Period Time, Min.	Pre-Injection Period Av. O_2 Used Cc./Min.	Post-Injection Period Time, Min.	Post-Injection Period Av. O_2 Used Cc./Min.	Remarks
M	139	20	90	4.0	90	3.5	L.A.[a]
M	140	20	120	4.2	105	4.0	L.A.
M	150	20	120	3.75	180	4.0	Quiet 60 min.; L.A. 120 min.
F	170	20	90	3.0	90	2.5	L.A. throughout
F	160	20	120	4.5	150	4.0	L.A. throughout
F	160	20	90	4.0	120	3.5	L.A. throughout
F	130	20	120	3.0	150	2.5	L.A. throughout
F	162	25	150	6.0	150	5.0	L.A. throughout
F	115	25	90	4.0	210	3.5	Quiet 60 min.; L.A. 150 min.
F	92	25	150	4.0	210	3.5	Good relaxation 30 min.; L.A. 180
F	154	25	150	5.0	150	4.0	Quiet 60 min.; Very L.A. 90 min.
F	110	25	150	3.25	210	2.75	Quiet throughout
F	85	25	150	2.50	150	2.0	Asleep 60 min.; L.A. 90 min.
F[b]	161	25	120	3.75	180	5.0	Excitement throughout
M[b]	146	25	150	3.50	110	4.5	Quiet 30 min.; Mild excit. 90 min.
F	110	25	150	3.5	150	3.25	Quiet 90 min.; L.A. 60 min.
F	123	25	120	3.5	240	3.0	Quiet 60 min.; L.A. 180 min.
F	186	25	120	6.0	150	5.0	L.A. throughout
F[b]	152	25	180	4.5	180	4.5	Mild excitation 90 min.; L.A. 90 min.
F	152	25	150	5.0	150	4.5	L.A. throughout
M[b]	120	25	120	5.25	120	6.0	Mild excitation throughout
F	157	25	120	4.75	120	4.25	Some excitation 60 min.; L.A. 60 min.
F	151	25	90	5.00	90	4.75	L.A. throughout
M	144	25	150	4.75	120	4.50	Quiet 30 min.; L.A. 90 min.
F	141	25	180	4.50	210	4.00	Quiet 30 min.; L.A. 180 min.
M	183	25	180	5.00	180	5.50	Excitation 30 min.; Awake 150 min.
M	180	25	120	5.25	210	5.00	L.A. 90 min.; Awake 120 min.
M	157	25	150	4.75	210	4.50	L.A. throughout
M	195	25	90	5.00	150	4.50	L.A. throughout
F	160	25	120	4.00	180	3.25	L.A. throughout

[a] L.A. = Light Anesthesia.
[b] These animals showed excitation rather than depression.

TABLE II.—COMPARISON OF EFFECTS OF "SECONAL SODIUM" INJECTIONS ON OXYGEN CONSUMPTION RATE IN MALE AND FEMALE RATS SEPARATELY AND IN BOTH GROUPS TOGETHER

No. of Rats and Sex	Dose of Seconal, Mg./Kg.	Average Oxygen Consumption		
		Pre-Injection, Cc./Min.	Post-Injection, Cc./Min.	Change, Cc./Min.
M— 3	20	4.00	3.83	−0.17
F— 4	20	3.63	3.13	−0.50
7 total	20	3.79	3.43	−0.36
M— 4	25	4.94	4.63	−0.31
F —14	25	4.36	3.77	−0.59
18 total	25	4.49	3.96	−0.53
M— 3	25	4.58	5.33	+0.75
F — 2	25	4.13	4.75	+0.62
5 total	25	4.40	5.10	+0.70
25	25	4.47	4.21	−0.26
30	20 and 25	4.50	4.00	−0.50

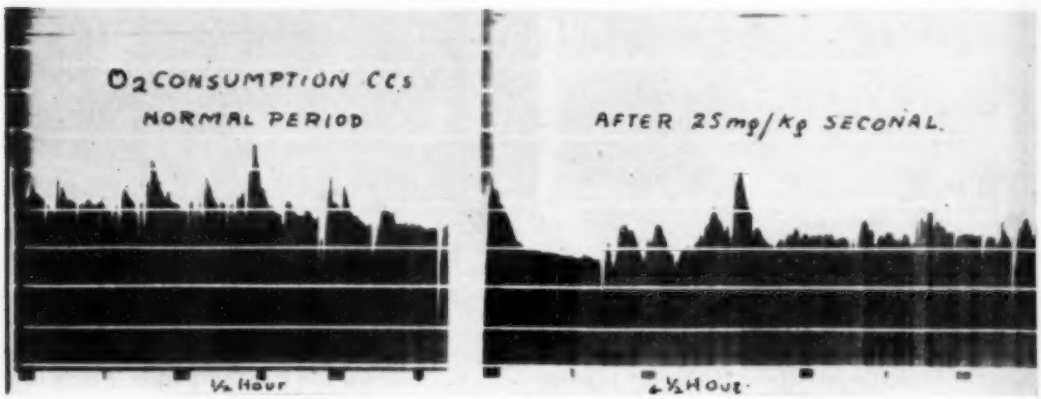

Fig. 3.—Showing that by a prolongation of the recorded observation periods, both for the control and experimental parts, reliable "average" rates of Oxygen Consumption can be obtained. Each black bar at the base represents one-half hour interval of time. The sharp peaks in the records are due to spontaneous periodic increases of activity of the experimental rats.

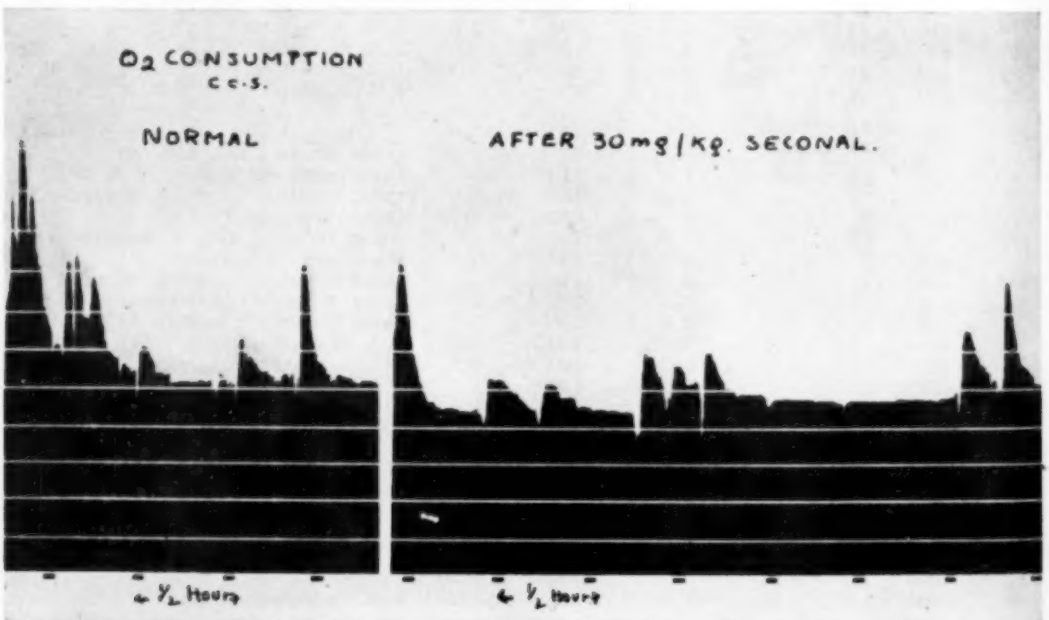

Fig. 4.—(See legend for Figure 3.)

in a few animals and thus increases their oxygen consumption rather than decreases it. We have found no marked changes in respiratory rates beyond those produced by the tranquilizing effect of the barbiturate. The rats showed sedation and light anesthetic effects of the drug. They could be easily aroused to activity by mechanical stimulation and they retained a good degree of skeletal muscle tone at the dosage levels indicated in Table I. The averages of the rate of O_2-consumption per minute of the various groups of animals are shown separately in Table II. In 5 rats included in this series, where Seconal produced slight excitation, the average O_2-consumption rate increased from a normal of 4.4 cc. to 5.10 cc. per minute. There is a greater degree of depression of oxygen consumption in the female rats than in the males.

CONCLUSIONS

In conclusion, the results indicate that in the majority of rats a significant reduction in oxygen consumption (metabolic rate) occurs when subanesthetic doses of Sodium Seconal are injected subcutaneously. In this paper the mechanism of the depressant effects of Seconal on oxygen consumption is not investigated. It also appears that Seconal, in the doses used, did not produce any changes in the respiratory rate and that muscular relaxation was not a contributory factor in the reduction of oxygen consumption under our experimental conditions.

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The Lipolytic Activity of Pancreatin U. S. P.*

By CHARLES W. BAUER† and EDWIN E. WILSON‡

A method of analysis which can be used successfully to determine the lipolytic activity of Pancreatin, U. S. P., is presented.

PANCREATIN U. S. P. contains the enzymes steapsin, amylopsin, and trypsin. The U. S. P. gives the minimum standards required for amylopsin and trypsin, but gives no minimum lipolytic activity. The only pharmacopœia requiring such standards is the British Pharmacopœia (4) which uses "the cream of fresh milk" as a substrate.

It appears that the reasons for not giving the lipase activity of Pancreatin, U. S. P. are the following:

(*a*) the inability to obtain a preparation with a large amount of lipase;

(*b*) the lack of a good assay; and

(*c*) the labile character of lipase.

The object of this investigation is to determine the lipolytic activity of Pancreatin U. S. P. and to find a method of assay whereby uniform results may be obtained. The degree of activity which is to be determined will not be based upon physiological conditions. This determination is impossible, since neither the length of time of the action of lipase in the animal body nor the influence of all the physiological activators or inhibitors has as yet been determined. For the same reasons minimum requirements given for trypsin and amylopsin in Pancreatin U. S. P. are not based upon the physiological action of these substances, and should not be so interpreted. It should be clearly understood that the degree of activity is merely determined under specific conditions suitable for analytical purposes, which may have little relationship to physiological conditions.

The literature of the biochemical journals gives numerous methods for the assay of lipase. A number of the better-known

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‡ This paper is based on a thesis presented to the Graduate Council of the Massachusetts College of Pharmacy by Edwin E. Wilson, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

methods of determination were tried and abandoned. The assay formulated by Willstätter, Waldschmidt-Leitz, and Memmen (1), with certain modifications, was most satisfactory for this investigation of U. S. P. Pancreatic lipolytic activity.

EXPERIMENTAL ANALYSIS

The Lipolytic Activity of Pancreatin U. S. P.—The determination of pancreatic lipase, as formulated by Willstätter, Waldschmidt-Leitz, and Memmen (1), is performed by titrating the liberated fatty acids produced as a result of the action of the fat-hydrolyzing enzyme steapsin, upon the substrate, olive oil, after digestion at 30° for one hour. The digestion mixture contains the enzyme preparation, ammonia-ammonium chloride buffer, calcium chloride, albumin, and olive oil. This reaction mixture is titrated in an alcoholic medium with 0.1 *N* alcoholic potassium hydroxide, using thymolphthalein as the indicator. The amount of 0.1 *N* alkali used, corrected for control, is the amount required by the liberated fatty acids. Willstätter and co-workers then converted the amount of alkali used into per cent of fat split. The per cent of fat split was then translated into lipase units. One Lipase Unit is the amount of enzyme required under the conditions specified above to split 24% of 2.5 Gm. of olive oil of a saponification value of 185.

If the splitting is more than 24%, the experiment should be repeated with one-half as much of the enzyme as was used before. If the splitting is less than 10%, the experiment should be repeated with twice as much enzyme. The percentage must be not less than 10% nor more than 24%; otherwise the lipase units cannot be determined. These limits have been incorrectly reported in many standard textbooks (3).

The Accuracy of the Assay Method of Willstätter, Waldschmidt-Leitz, and Memmem.—The digestion mixture of Willstätter, composed of several water-insoluble substances, fails to give a reaction that is uniform for the entire period of digestion. The presence of a water-insoluble substrate would seem to militate against uniform digestion, and this assumption was confirmed experimentally. Upon repetition of the experiment, a series of divergent readings was obtained. The average titration value was 12.76 cc. The standard variation computed from these results was 1.12 cc. The presence of this sizable variation suggested the search for a satisfactory inert emulsifying agent which could be used to get more nearly uniform reactions and consequently more nearly comparable titration values.

The Value of Bentonite in the Analysis of U. S. P. Pancreatic Lipolytic Activity.—After a search for inert emulsifying agents which would give more nearly homogeneous reaction mixtures, bentonite was found to be the most satisfactory substance tried. The use of a blank determination ruled out any effects that bentonite might have upon the reagents which could possibly lead to an erroneous interpretation of enzymatic activity.

Three cubic centimeters of Magma of Bentonite N. F. was used in place of the 3 cc. of water used by Willstätter. The use of bentonite reduced the standard variation from 1.12 cc. to 0.27 cc.

The Amount of Pancreatin U. S. P. That Can Be Used to Determine Its Lipolytic Activity by the Willstätter Method in the Presence of Bentonite.—It was found that more than 400 mg. of Pancreatin U. S. P. in the presence of bentonite failed to give uniform results. This was also true in the absence of bentonite. Any amount between 200 and 400 mg. of Pancreatin U. S. P. analyzed gave results from which a satisfactory and uniform curve could be plotted.

The Effect of Bile Salts on the Titration Values in the Presence of Bentonite.—Bile salts produced a significant lipase activation when added to the digestion mixture at the start, if the medium was alkaline; but the yellow color imparted to the reaction mixture, because of the bile salts, made it very difficult to get a sharp end point. Since a sufficient degree of lipase activity is obtained without the use of bile salts, and since the end point is more readily determined in the absence of them, it was deemed advisable to determine U. S. P. lipase activity without making further use of this impure activator.

The Abandonment of Albumin as an Activator.—The influence of albumin on lipase activity is believed to be something more than that of activation. Willstätter used albumin to compensate for the inhibitory effect of certain unidentified substances found in pancreatic preparations. The use of albumin for this purpose is subject to criticism. In the first place, it is difficult to get two samples of albumin of identical composition. In the second place, Pancreatin U. S. P. contains a proteolytic enzyme which acts upon albumin to form acids which influence the titration value materially (2). Since the lipase activity is based upon the amount of alkali required to neutralize the acids produced as a result of lipolytic activity, every precaution must be taken to avoid the formation of acids in any other way.

The titration values of lipase activity were sufficiently high in the absence of albumin to warrant its elimination, and the acidity so obtained is a true index of the amount of fatty acids produced as a result of pancreatic lipolytic activity.

The standard variation for the titration values in the presence of albumin and bentonite was found to be 0.27 cc. In the absence of albumin but in the presence of bentonite it was found to be 0.19 cc.

The Effect of Various Amounts of Bentonite on Lipolytic Activity of Pancreatin U. S. P. in the Absence of Albumin.—It appears that from 3 to 5 cc. of Magma of Bentonite N. F. produces the best results for analysis in the absence of albumin, and that this amount of bentonite has no appreciable effect upon the enzyme. The chief value of the bentonite is the production of a uniform mixture, by

means of which more of the fat is brought into contact with the enzyme, and which consequently gives a higher titration value in a given period of time.

The Amounts of Pancreatin U. S. P. That Can Be Used to Determine Its Lipolytic Activity in the Absence of Albumin but in the Presence of Bentonite.—It was found that more than 400 milligrams of a sample of Pancreatin U. S. P. could not be used if the lipase content were to be determined by the Willstätter Method. It was thought to be advisable to determine the amount of Pancreatin U. S. P. that could be assayed by this method in the absence of albumin but in the presence of bentonite. Here, too, it was found that 400 mg. was the upper limit that could be used to make this determination.

A Comparison of the Different Methods Used to Determine the Lipolytic Activity of a Sample of Pancreatin U. S. P.—Bentonite favors the formation of a finely divided substrate, giving a higher degree of digestion and more nearly uniform results. It appears that albumin either activates the lipase, neutralizes the action of some inhibitory substance, or serves as a substrate for the proteolytic enzymes in pancreatin. The increase in acidity may be due to either one or all of these three actions of albumin. A comparison of the data obtained by different methods of assay is shown in Table I.

TABLE I.—A COMPARISON OF PANCREATIC LIPOLYTIC ACTIVITY OF DIFFERENT METHODS OF ASSAY

Method of Analysis Used	Cc. of 0.1 *N* Alcoholic Potassium Hydroxide
Willstätter	10.66
	10.91
	8.31
	20.81
Willstätter (with bentonite)	20.00
	20.00
	17.36
Willstätter (with bentonite but without albumin)	17.21
	17.84

The Influence of Ether on the Lipolytic Activity of Pancreatin U. S. P.—In a series of experiments, 10 Gm. of Pancreatin U. S. P. was shaken intermittently with 500 cc. of ether for twelve hours at room temperature. The suspension was filtered and both the filtrate and residue were assayed. The ether-soluble fraction showed no lipolytic activity.

The ether insoluble fraction showed the same degree of lipolytic activity as that of an equal amount which had not been treated with ether. These results indicate that pancreatic lipase is neither soluble nor is it destroyed to an appreciable extent by ether. This finding leads one to question the use of ether for the purpose of stopping lipolytic activity as is done in the Willstätter Assay. The data of these findings are shown in Table II, and contradict the literature on the subject (3), which states that ether stops the action of lipase.

In a series of digestions, carried out as in previous experiments, the digestion mixture was washed with

TABLE II.—THE LIPASE ACTIVITY OF THE ETHER INSOLUBLE FRACTION OF PANCREATIN U. S. P.

Sample Analyzed	Mg. of Pancreatin Used	Cc. of 0.1 *N* Potassium Hydroxide
Untreated sample	300	16.07
	300	16.07
Ether insoluble fraction	300	15.06
	300	14.35

alcohol into a titration flask containing 20 cc. of ether. The mixture was made up to a volume of about 125 cc. with alcohol, and was titrated with 0.1 *N* alcoholic potassium hydroxide. In the control flasks, numbers 4, 5, and 6, (Table III), the pancreatin was added just before the titration. Flasks 1, 2, and 3 were titrated one hour after adding the pancreatin and were then allowed to sit for forty-eight hours at room temperature and again titrated. At the end of forty-eight hours additional potassium hydroxide was required to neutralize the acid which had been formed during this time. Flasks 7 and 8 were not titrated until they had sat for forty-eight hours at room temperature.

From these titration values it may be seen that the ether does not destroy the lipase activity of Pancreatin U. S. P. If, on the contrary, ether destroyed the lipase action then the readings should have been no greater for flasks 7 and 8 than the total titration values given for numbers 1, 2, and 3. These data suggest that the retarding effect in the first three flasks were due to either the alcoholic potassium hydroxide or the *p*H of the solution. It is obvious from these findings that ether may retard, but it does not destroy, the lipolytic activity of Pancreatin U. S. P.

TABLE III.—THE EFFECT OF ETHER ON LIPOLYTIC ACTIVITY

Flask No.	Mg. of Pancreatin	Cc. of Alkali after 1 Hr. Digestion	Cc. of Alkali 48 Hr. after Adding Ether	Total Cc. of 0.1 *N* Potassium Hydroxide
1	300	54.6	3.2	57.8
2	300	55.0	5.1	60.1
3	300	54.3	3.2	57.5
4 (control)	300	33.4	1.2	34.6
5 (control)	300	33.9	1.5	35.4
6 (control)	300	34.1	1.5	35.6
7	300	..	84.1	84.1
8	300	..	82.5	82.5

The Lipolytic Activity of Pancreatin U. S. P. in the Absence of a Buffer, but in the Presence of Bentonite.—Since bentonite was found to be an efficient emulsifying agent whose action as a buffer has not been tested, it was felt that it might serve a double purpose and that the ammonia-ammonium chloride buffer might advantageously be eliminated. However, the titration values obtained in the absence of the ammonia-ammonium chloride buffer were too low for advantageous elimination.

An Improved Method of Analysis for the Determination of Pancreatic Lipase.—Into a glass-stoppered 50-cc. Erlenmeyer flask, introduce exactly 2.5 Gm. of Olive Oil U. S. P., 3 cc. of Magma of Bentonite N. F., 0.5 cc of 2% calcium chloride solution, and 2 cc. of buffer (0.66 cc. of normal ammonium hydroxide and 1.34 cc. of normal ammonium chloride). Rotate this mixture several times so as to produce a thick emulsion. Make the enzyme preparation containing 300 mg. of pancreatin, up to 10 cc. with distilled water; add this preparation to the flask and agitate vigorously for one minute so as to emulsify thoroughly the mixture. Place this reaction mixture in a constant temperature bath adjusted to 30°. Exactly one hour after the introduction of the enzyme preparation, remove the flasks and the controls, wash their contents with Alcohol U. S. P. into larger flasks containing 20 cc. of ether so as to make a total volume of 125 cc. Add twelve drops of 1% thymolphthalein and titrate immediately with 0.1 *N* alcoholic potassium hydroxide.

Do not add the enzyme preparation to the control flasks until they are removed and ready for titration. Subtract the titration of the control determination from the enzyme-digestion mixture in order to get the amount of alkali that is used to neutralize the fatty acids as a result of lipolytic activity.

The Lipase Activity of Commercial Samples of Pancreatin U. S. P.—To determine the lipase content of commercial samples of pancreatin, samples were obtained from different drugstores. These preparations were manufactured by three different pharmaceutical houses from whom was ascertained the exact age of each sample. These samples had been opened and stored under various conditions. Some samples were powdery, light-cream-colored preparations, while others were caked and were dark brown in appearance. These samples were assayed by the method given above. The analyses indicate that the age of the sample is not the factor most significant of its lipolytic activity. The data in Table IV show that some of the older samples contained almost as much lipase as did the more recent.

TABLE IV.—THE LIPASE ACTIVITY OF 300-MG. SAMPLES OF PANCREATIN COMING FROM VARIOUS MANUFACTURERS

Sample No.	Company	Age in Mo.	Cc. of 0.1 *N* Potassium Hydroxide
1	A	15	17.966
2	B	15	26.455
3	B	13	27.800
4	C	72	23.075
5	C	36	24.000
6	B	180	8.700
7	B	>180	1.050

The analyses seem to indicate that Pancreatin U. S. P. contains enough steapsin to be included in the minimum requirements of the official definition. An official method of assay similar to the one used for these assays could then be adopted.

RECOMMENDATION

The data obtained in this investigation suggest the possibility of introducing the following paragraph into the official definition of Pancreatin U. S. P. (5).

The potency of Pancreatin U. S. P. shall be such that when assayed as directed it shall convert not less than its own weight of Olive Oil U. S. P. into fatty acids in one hour.

The adoption of this paragraph would also require the introduction of an assay method, such as has been recorded in this thesis, into the official monograph of Pancreatin U. S. P.

SUMMARY

1. Pancreatin U. S. P. has a measurable amount of lipolytic activity according to Willstätter's method of analysis.

2. The results of the assay of Pancreatin will vary in accordance with its age and method of analysis, and probably with the method of manufacture and storage.

3. Bentonite is an inert emulsifying agent which is very useful in the determination of lipolytic activity.

4. Ether appears to have no effect upon lipolytic activity and may not be used to stop the reaction. However, the ether should be retained in the assay, because it dissolves the undigested olive oil and hence gives a clearer end point.

5. The color produced by bile salt preparations makes them undesirable activators in this determination.

6. Albumin should not be used in this assay since it is acted upon by the proteolytic enzymes present in the pancreatin.

7. A method of analysis has been worked out which can be used successfully to determine the lipolytic activity of Pancreatin U. S. P.

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A Critical Investigation of the Amylolytic Activity of Pancreatin and Extract of Malt*

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The age of the product does not determine its amylolytic activity. A good appearing pancreatin eleven years old had a higher amylase activity than an off-colored, but recently manufactured, product. The official definitions of pancreatin and extract of malt are misleading. The official definitions should give the time involved in the assays in order to give a truer picture of their enzymatic activities.

KIRCHHOFF (in 1814) was the first to record the effect of a biochemical catalyst when he observed the influence of the glutinous component of wheat meal upon the conversion of starch to soluble carbohydrates. This phenomenon was later classified as enzymatic activity by Kühne (1878). A great deal of work has been done in determining amylolytic activity of substances since that time; yet the official method recorded in the United States Pharmacopœia for the amylase activity of Pancreatin and Extract of Malt is still subject to considerable criticism.

In order to evaluate the official assay method now in use a critical investigation of the substrate and its preparation, of the period of digestion, of the end point, of the temperature, and even of the official definition should be made. Starch, without indicating its source, is the conventional substrate used in most qualitative and quantitative assays. The Pharmacopœia specifies potato starch. Since the chemistry of the starches is not entirely understood, and since there are individual differences which manifest themselves in digestion experiments, the choice of a particular starch in the Pharmacopœia may be looked upon as worth while. The use of potato starch is based upon the findings of Graber (1). The theory that starches are made up of different components in varying proportions, some of which are attacked by one group of amylases and some by others, makes the choice of the substrate even more important. Without specifying any botanical variety, Van Klinkenberg (2) asserts that starch is a mixture of 36 per cent α-starch and 64 per cent β-starch. Kühn (3) does not subscribe to the "mixture theory." He believes that starch is a single molecule with a chain of alternating α and β glucosidic linkages. Here again, the nature of the amylase will determine whether it will attack the α or the β linkage which will materially influence the nature of the intermediary products.

It has been fairly well established that starch consists of two substances, α-amylose or amylopectin, found in the outer layer of the granules and not readily dispersible in water, and β-amylose found in the interior and dispersible in water. Various workers have separated these two components and have reported yields of soluble amylose varying from 17 per cent to 98 per cent depending upon the method of separation. According to Taylor and Iddles (4) the yield of α-amylose depends upon the botanical source of the starch. Potato starch gave the lowest yield of those varieties tried. This may explain why potato starch, according to Graber (1), is more readily converted into soluble carbohydrates by pancreatic amylase than are the other starches.

Kühn (3) made the important observation that pancreatic and salivary amylases act upon starch to give primarily α-maltose. He classified these enzymes as the *alpha* type. He named another group of amylases, obtained from sprouted and unsprouted grains, the *beta* amylases, since they act upon the same substrate to give a large yield of β-maltose.

Various methods are used for the estimation of amylolytic activity. Chief among these are:

1. Measurement of the reducing sugar

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formed as a result of the digestion of starch by an amylase.

2. Measurements of the decrease in viscosity of the reaction mixtures.

3. Observations of the changes in color given by the reaction mixtures when treated with iodine.

4. Determination of residual amyloses.

The United States Pharmacopœia recognizes the third method for the quantitative determination of amylase in Pancreatin and Extract of Malt. An important objection to this method of analysis is that the assay methods use different intervals of time for the digestion period. Although the interval of time is given in each of the respective assays, it is not indicated in the official definition of either. The quantitative values given in the official assays are indeed misleading.

The object of this investigation is to find a more satisfactory method of analysis for amylase activity than that given by the United States Pharmacopœia, and to suggest changes in the monographs for Pancreatin and Extract of Malt which will avoid the misinterpretations which are now commonly made. The present monographs lead many to believe that Pancreatin contains about five times as much amylase as Extract of Malt, but the true ratio is about 30 to 1.

EXPERIMENTAL

The potato starch paste made by the official method either under Pancreatin or Extract of Malt is a heterogeneous mixture which does not give uniform digestion. The color of the end point of the assay is very difficult to read accurately, and the error is thus magnified. Furthermore, no consideration is given to the influence of such factors as activators or the control of hydrogen ion concentration.

It was found that a more nearly homogeneous starch paste could be obtained if, after being boiled, it was run through a hand homogenizer several times. The homogenized paste is less viscous than the official paste. A small amount of starch is lost in the homogenizing, which can be corrected by adding 1% of potato starch to the original amount. If this is done the loss is negligible (about 0.1%).

An alternate method is to make a paste by boiling 5.625 Gm. of dry starch for five minutes with 125 cc. of distilled water in a tared beaker of 250 cc. capacity. It is cooled to room temperature and enough distilled water is added to make the mixture weigh 150 Gm. One hundred grams of this paste (containing 3.175 Gm. of dry starch) is used.

In the official assay of amylase activity on potato starch, the end of the reaction is indicated by the absence of a blue or violet color between the iodine and the starch digestion mixture. The starch digestion mixture reacts with iodine to give colors which vary from blue to violet, to various shades of red and pink; and as digestion proceeds they finally fade to the color of the standard iodine solution which is employed. The temperature of the iodine solution must be carefully controlled, since varying the temperature not only affects the time required to reach the official end point, but gives color variations peculiar to the temperature employed.

The assays were run according to the directions given in the United States Pharmacopœia with different temperatures of the iodine solution in order to determine the influence of this factor.

It was found that the temperature of the iodine test solution plays an important role in determining the end point. The tests were made at fifteen-second intervals.

The standard used was the blue solution obtained by adding 0.1 cc. of the digestion mixture (0.15 Gm. of pancreatin added to 3.75 Gm. of dry starch made into a paste and digested at 40° for five minutes) to the dilute iodine solution maintained at 24.8°.

At 12° the starch iodine solution showed a distinctly bluish green color which tended to go to blue. At 25° the solution was a decided blue, with a tendency toward purple as the digestion continued. At 38° the solution, even during the first few minutes of digestion, showed a red color which gradually faded.

Table I shows the digestion time that is necessary in order to get the color with iodine that matches the standard.

TABLE I.—SENSITIVITY OF THE STARCH IODINE REACTION TO TEMPERATURE CHANGE

No.	°C. of Reaction Mixture	°C. of Iodine Solution	Period, in Min., Digestion in Order to Reach the End Point
1	40	12.5	6.5
2	40	15.3	6.0
3	40	18.6	5.75
4	40	24.8	5.0
5	40	32.0	3.5
6	40	38.5	1.5

The United States Pharmacopœia states that the temperature of the iodine test solution should be "23° to 25°." It is apparent that this temperature is important and must be maintained for comparative work, for any variations in the temperature of the iodine solution will materially influence the results.

It was found to be very difficult to match the colors considered to be the true end point. However, the intensity of the colors could be compared satisfactorily.

The sensitivity of the homogenized starch iodine test was determined as follows: 1.0 Gm. of dry starch, accurately weighed, was made into a paste by boiling with 100 cc. of distilled water for five minutes. The paste was cooled to room temperature and homogenized, and distilled water was added to make 250 cc. Various dilutions of this preparation were made and treated with the dilute iodine solution. The tests were made at room temperature (23°). The dilute iodine solution was made according to the Pharmacopœia and consisted of 0.2 cc. of 0.1 *N* iodine in 60 cc. of distilled water.

While the starch iodine reaction could be detected in dilutions of 1 to 20,000 if observed instantly at the point of contact, it could not be detected when the two solutions were mixed as directed to be done in the Pharmacopœia. In fact, 1 part of starch in 2000 parts failed to give a positive test.

Thus a considerable quantity of starch may be undigested and still fail to manifest itself by the official method of identification.

Starch which had not been homogenized was also used. The difficulty of getting uniform dilutions and even distribution of the color produced made it impossible to record comparative values.

The Effect of Light on the Starch-Iodine Color.—Willson (5) suggested that a reference sample of U. S. P. Pancreatin be chosen as a standard, and that the assays of unknown pancreatin preparations be compared with it. It is important to note the influence of light upon any color standard of this character.

A typical blue color of the starch-iodine reaction was obtained by adding a small portion of a partially digested starch paste to the dilute iodine solution. This blue solution was divided into three portions.

Portion A was placed directly in the sunlight. Portion B was kept in the room away from the direct rays of the sun but in diffused light. Portion C was kept in a dark locker when it was not employed as the standard.

A comparison of portion C at the end of two hours with portion A at the end of the same period of time proved clearly that the color fades very greatly in direct sun light. This is true to a lesser extent in diffused light, yet it is significant in quantitative determinations, and indicates that the standard should not be made too far in advance.

Determination of a Satisfactory End Point.—Sherman, Kendall, and Clark (6) determined the end point of starch digestion by using larger amounts of digestion mixture and iodine in a smaller volume than in the official assay. This intensifies the color before the end point is reached and makes the end point, where there is "no color which can be distinguished from that of the untreated iodine solution," more definite.

Experiments were made to determine if this end point could be used in the official assay of Pancreatin with the amounts of digestion mixture and iodine solution called for by the Pharmacopœia.

The use of larger quantities of starch than directed in the Pharmacopœia was also tried to determine its value over a wide range of concentrations. Each succeeding assay contained 3.75 Gm. of additional dry starch diluted in the same ratio as before, with an increase in weight of 100 Gm. over that used in the preceding assay.

Digestion was considered complete, as directed in the Pharmacopœia, when "0.1 cc. of the digestion mixture, added to a previously made mixture of 0.2 cc. of 0.1 *N* iodine in 60 cc. of distilled water," showed as indicated by Sherman, Kendall, and Clark (6) "when viewed against a white background, no color which can be distinguished from that of the untreated iodine solution." This is the Sherman, Kendall, and Clark (6) end point using concentrations of digestion mixture and iodine solution called for by the Pharmacopœia.

TABLE II.—DETERMINATION OF THE TIME REQUIRED FOR THE DIGESTION OF VARYING AMOUNTS OF STARCH USING A MODIFIED SHERMAN, KENDALL, AND CLARK (6) END POINT

No.	Mg. Pancreatin	Gm. Dry Starch	Total Wt. of Mixture, Gm.	Time, in Min., Required for Digestion
1	150	3.75	100	8
2	150	7.50	200	20
3	150	11.25	300	36
4	150	15.00	400	50
5	150	18.75	500	61
6	150	22.50	600	75
7	150	26.25	700	101
8	150	30.00	800	109

It will be recalled that in the official method the end point is considered reached when no blue or violet color is produced, but the allowance of some color makes it very indefinite.

It takes the digestion mixture a longer period of time to attain the end point of Sherman, Kendall, and Clark (6) than that given in the Pharmacopœia for its end point. However, it was found that the eight minutes required to reach the end point by this method is comparable to the amount of Pancreatin which takes five minutes by the method of the United States Pharmacopœia, and is much easier to see.

The experimental data giving the time required to reach this end point not only by using the amount of starch indicated in the Pharmacopœia but in greater concentrations are recorded in Table II, from which it appears that reaction number 1 is the best to use.

A Modification of the Official Assay.—Upon the strength of the experimental evidence concerning the use of homogenized starch, and the modification of the Sherman, Kendall, and Clark (6) end point, the following changes were made in the official assay:

Instead of 3.75 Gm. directed in the official assay, 3.788 Gm. of homogenized potato starch was used.

The total weight of the mixture was increased from 100 to 150 Gm. to allow for the rinsing of the homogenizer.

Water, previously heated to boiling, instead of the temperature of 50° to 60° was found to hasten the gelatinization of the starch and to prevent the formation of a tough paste at the bottom of the beaker. This paste is not only difficult to homogenize, but is not readily digestible.

The modified end point of Sherman, Kendall, and Clark (6) was employed. The italicized portion of the following monograph indicates the extent to which the official assay was modified.

"Determine the percentage of moisture in potato starch by drying 0.5 Gm. accurately weighed at 120° for four hours. Thoroughly mix a quantity of the starch, equivalent to *3.788 Gm. of dry starch (3.75 Gm. plus* 1%) with 10 cc. of cold distilled water. Add the mixture with constant stirring to 75 cc. of distilled water, *previously heated to boiling*, contained in a 250 cc. beaker. Rinse the remaining starch into the beaker with 10 cc. of distilled water, and boil it gently for five minutes or until a translucent uniform paste is formed. Cool to room temperature. *Run it through a hand homogenizer three times, collecting it in a tared 250 cc. beaker. Pour several 20 cc. portions of hot distilled water into the homogenizer, in order to remove the remainder of the starch paste, and add the rinsings to the paste in the beaker. Add enough distilled water to make the mixture weigh 150 Gm.*

"Warm the paste to 40° and place the beaker in a water bath maintained at 40°. Suspend 0.15 Gm. of pancreatin in 5 cc. of distilled water and add the suspension to the starch paste, mixing it well by pouring the mixture from beaker to beaker for 30 seconds, noting the time when the pancreatin suspension was added to the starch. Maintain the mixture at 40° for exactly *eight* minutes. At once add 0.1 cc. of the starch pancreatin mixture into a previously made mixture of 0.2 cc. of 0.1 *N* iodine in 60 cc. of distilled water. *When viewed against a white background, there should be no color which can be distinguished from that of the untreated iodine solution.*"

The Preparation of β-Amylose.—Sherman and Baker (7) found that pancreatic amylase produced sugar more rapidly from β-amylose than from α-amylose. They also found that in the early stages of digestion malt amylase showed a greater yield from α-amylose than from β-amylose.

In order to see if any appreciable difference in the activity of pancreatic amylase could be obtained by using β-amylose in place of whole starch, a method of obtaining this component was needed. The literature gives various methods which are employed for the separation of the components of potato starch.

Gruzewska (8) effected a separation by treating potato starch with dilute alkali, and after neutralization and sedimentation, decanting the dissolved amylose from the residue of amylopectin. The amylose represented 40–45% of the original starch.

By treatment of potato starch with hot water, sedimentation, and decantation, Tanret (9) estimated 27% of amylose.

Sherman and Baker (7) subjected a thin paste of potato starch to centrifugal force, and obtained about 15% in *beta* amylose form.

Samec and Mayer (10) *electrodialyzed* a dispersion of potato starch prepared at 120°. The amylose fraction represented 17%.

Ling and Nanji (11) *fractionated* potato starch by freezing a paste, and after melting the frozen mass, separated the solution of amylose from the amylopectin by centrifuging.

Taylor and Iddles (4) disintegrated starch grains, previously treated with hydrogen chloride in alcohol, with ammonium thiocyanate solution, then precipitating with alcohol and subjecting the precipitate to ultra-filtration or dialysis. The yield of soluble amylose was 97–98%.

Baldwin (12) effected separation by gelatinizing, freezing, and performing a series of extractions.

A rapid and satisfactory method was found which consisted of boiling a 2.5% starch paste for thirty minutes to rupture the granules. The paste was then precipitated with alcohol, dried, and powdered. The soluble amylose was separated from the insoluble component by allowing to stand in cold water, decanting, and boiling the aqueous solution to concentrate the β-amylose. The insoluble α-amylose was washed free of β-amylose, precipitated, dried, and powdered. A yield of about 36.5% of β-amylose was obtained.

A Detailed Method of Separating Potato Starch into Alpha and Beta Amylose.—Thoroughly mix a quantity of starch equivalent to 10.0 Gm. of dry starch U. S. P. with 50 cc. of cold distilled water, and add, with constant stirring, to a sufficient amount of boiling distilled water to make 350 cc. Rinse the remaining starch into the beaker with two 25-cc. portions of distilled water and boil gently with constant stirring for thirty minutes. Add distilled water when necessary to maintain the original volume. Allow the paste to cool to room temperature. Transfer the paste to a 2000-cc. Erlenmeyer flask, using 50 cc. of distilled water to remove the remainder of the paste from the beaker. Now superimpose over the starch paste 500 cc. of 95% alcohol, stopper the flask and shake vigorously until the starch is completely precipitated. Filter and allow the precipitate to dry for twelve hours at 65°. Grind the dry altered starch in a mortar, and pass the powder through a number 100 sieve.

Weigh about 5 Gm. of the altered starch and sprinkle it with constant stirring into 500 cc. of cold distilled water in a 2000-cc. beaker. Add enough water to this mixture to make the final volume measure about 1000 cc. and let it settle for twenty-four hours. Decant the clear supernatant liquid and concentrate it by boiling to a volume of about 200 cc. or until the β-amylose will be precipitated with an equal volume of alcohol. Precipitate the β-amylose with 200 cc. of 95% alcohol and dry it at

65°. Powder the precipitate so that it will pass through a number 100 sieve. This was used as the β-amylose in succeeding work.

Saccharogenic Activity of Pancreatin Using Different Substrates.—In addition to β-amylose, potato starch, corn starch, and soluble starch were used.

The method of analysis used was based on the amount of maltose produced from a given amount of substrate in a given unit of time. It is essentially the method of Willstätter, Waldschmidt-Leitz, and Hesse (13). These authors used soluble starch as the substrate. The different substrates being tested were prepared by making one per cent pastes and boiling for five minutes.

It was found that the different substrates gave no appreciable difference in the amount of maltose produced. There appears to be no advantage in using β-amylose for this determination. If any conclusions could be drawn from these findings, the chief one would be that β-amylose is slightly inferior to the other substrates used. The experimental data are recorded in Table III.

TABLE III.—SACCHAROGENIC ACTIVITY OF PANCREATIN USING VARIOUS STARCH SUBSTRATES

Substrate	Mg. Pancreatin	Cc. 0.1 *N* Iodine	Mg. Maltose
Homogenized potato starch	5	4.09	70.14
Potato Starch	5	4.15	71.17
Corn Starch	5	4.27	73.23
Soluble Starch	5	4.07	69.80
β-Amylose	5	3.40	58.31

The Amylase Activity of Different Samples of Pancreatin.—During the course of the experiments it was noted that many of the older samples of pancreatin had caked and had assumed a darker color than usual. Experiments were made to compare the amylolytic activity of the older samples with several fairly fresh samples of pancreatin.

In order to determine accurately the loss in amylolytic activity due to aging, it would be necessary to store a sample of pancreatin under a certain set of conditions, and to determine its activity from time to time. Since this was impractical, several commercial samples of pancreatin were obtained from different drugstores in the vicinity. These samples varied in age from eleven months to fifteen years and came from three different pharmaceutical houses. The conditions under which the samples were stored no doubt varied considerably, since some of the samples were typical cream-colored powders, while other samples were caked, and of a deep brown color.

The activities of these samples of pancreatin were tested by the method of Willstätter, Waldschmidt-Leitz, and Hesse (13). The only change from the original assay was that a 1% homogenized potato starch paste, which had been boiled for five minutes, was used in place of the soluble starch suggested by the authors.

One hundred milligrams of pancreatin was suspended in 100 cc. of distilled water, and 5 cc. of this suspension was added to the substrate.

The pancreatin assayed by the above method showed an irregular drop in amylase activity as the ages of the samples increased. These findings fail to establish any reasons for the irregularity in amylase content. It appears that the age of the sample is of minor consequence, since properly stored samples, judged by their physical appearance, of 16, 53, 60 106, and 132 months of age had practically the same enzyme activity. The samples which were caked and brown in color gave a lower amylolytic activity. Whether these samples were inferior to begin with or changed in appearance and amylolytic activity because of improper storage could not be determined. Nevertheless, it appears that Pancreatin which has caked or is dark brown in color does not have the amylolytic activity which is found in the dry, powdery, light-cream-colored samples.

The experimental work is tabulated to show the source of pancreatin, as derived from companies "A," "B," and "C," and may be seen in Table IV.

TABLE IV.—DETERMINATION OF THE AMYLASE ACTIVITY OF SAMPLES OF PANCREATIN FROM DIFFERENT COMPANIES

Co.	No.	Age Mo.	Cc. 0.1 *N* Iodine	Mg. Maltose
A	1	39	4.53	77.69
A	2	53	4.68	80.26
A	3	92	4.58	78.55
A	4	98	5.58	78.55
B	5	60	4.47	81.29
B	6	60	4.74	81.29
B	7	96	4.69	80.43
B	8	120	3.14	53.85
B	9	132	4.64	79.58
B	10	156	4.02	68.94
C	11	15	3.55	60.88
C	12	16	4.74	81.29
C	13	28	3.24	55.57
C	14	47	3.19	54.71
C	15	69	4.12	70.66
C	16	82	4.58	80.55
C	17	133	0.67	11.49
C	18	142	0.31	5.32
C	19	180	0.31	5.32

SUMMARY

1. The heterogeneous character of the potato starch paste in the United States Pharmacopœia for the assay of amylase activity under Pancreatin makes it very difficult to get uniform reaction mixtures. This condition may be greatly improved by homogenizing the starch paste.

2. The starch iodine end point of the official assay is unsatisfactory for the following reasons:

(*a*) One part of starch in a 2000 dilu-

tion cannot be differentiated from the standard iodine solution.

(*b*) The starch iodine reaction is very sensitive to light.

(*c*) The temperature of the iodine solution not only influences the color and intensity, but also the time involved in reaching the end point.

(*d*) The end point is not a true indicator of amylase activity, since it does not register the termination of this hydrolytic reaction.

3. Pancreatic amylase is relatively stable over long periods of time when properly stored. Samples of good appearance varying in age from sixteen months to eleven years had practically the same enzyme activity. Other samples of similar age but of poor appearance had a much lower activity.

4. If the official assay is retained, it is recommended that the following modifications of the official monograph of Pancreatin be made:

(*a*) The starch paste used should be homogenized. There is no good reason to insist on the use of potato starch.

(*b*) The wording of the end point "*no blue or violet color is produced*" be changed to *no color is produced which can be distinguished from that of the untreated iodine solution.*

(*c*) The official definition of amylase activity, wherein it states "*Pancreatin converts not less than 25 times its weight of starch into soluble carbohydrates,*" be modified by adding the phrase, "*in 5 minutes.*"

(*d*) It is further suggested that these modifications be observed in the official assay of Extract of Malt and that the definition of amylase activity for this preparation be modified to read, *Extract of Malt is capable of converting not less than five times its weight of starch into water soluble sugars in 30 minutes.*

5. The modifications suggested in 4 *c.* and *d.* should be adopted in order to avoid the misinterpretation which is commonly made because the time element is not conspicuously recorded.

6. The substitution of different starches or components of starch for the soluble starch by the method of Willstätter, Waldschmidt-Leitz, and Hesse (13) gave practically the same pancreatic amylolytic activity. This is an advantage over the official method which specifies the use of *potato starch.* Furthermore the use of activators, the control of *p*H, and the determination of the amount of maltose as the end product give this method added advantages over the official method.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

l-Ornithine
Sparassol
6-Methylsalicylic acid
Papaveraldine
Laudanosine
Codamine
Carnegine
Pellotine
d- and *l*-Arabitol
Trihydroxyglutaric acid
Phosphopyruvic acid
dl-Phenylglyceric acid
Pregnanediol
Isoandrosterone
Dehydroandrosterone
2-Aminobutyrolactone hydrobromide
5-Chloropentanone-2
Emetine
Dimethylhydroxylamine
d- and *dl*-Cysteine hydrochloride

A Study of the Tryptic Activity of Pancreatin U. S. P.*

By CHARLES W. BAUER† and ARTHUR K. WHITE‡

The official assay for pancreatic trypsin is not an assay for a single enzyme but for a mixture of proteolytic enzymes. Many samples labeled Pancreatin U. S. P. possess three times the minimum U. S. P. trypsin requirements. Triple-strength pancreatin is frequently no stronger than ordinary U. S. P. Pancreatin.

PANCREATIN is described in the United States Pharmacopœia as "a substance containing enzymes, principally pancreatic amylase, trypsin, and pancreatic lipase, obtained from the fresh pancreas of the hog .....". Without mentioning trypsin directly, but only by inference, the monograph further states that "pancreatin converts not less than 25 times its weight of casein into proteoses."

"Trypsin" is the name coined by Kühne (1) in 1867 for a proteolytic enzyme found in pancreatic juice. It means "a substance that cleaves." Kühne also used the name "Pancreatin" for the trypsin-containing precipitate that he obtained when he treated the pancreatic extract with alcohol.

The proteolytic effect of pancreatic juice was first recognized by Purkinje and Pappenheim (2) as early as 1836. A more comprehensive study than theirs of the effect of pancreatic juice on egg albumin was made by Corvisart (3) in 1856. However, it remained for Kühne (1) and his co-workers to recognize the fact that the proteolytic enzyme or enzymes in pancreatic juice carried the decomposition of egg albumin beyond the peptone stage. They found that leucine and tyrosine were among the products of decomposition when peptones were treated with either pancreatic juice or minced pancreatic extract.

Northrop and Kunitz (4) obtained a crystalline proteolytic enzyme from the pancreas of cattle in 1932. They retained the name "Trypsin" to designate "the most important proteinase" found in the pancreas.

The trypsin found in Pancreatin U. S. P. is the proteolytic enzymes, that convert a special preparation of casein into decomposition products that are not precipitated by a specially prepared mixture of acetic acid and alcohol. The official assay is based upon the formation of proteoses by this process; but since trypsin, as Kühne (1) has shown, actually converts proteoses into simpler compounds, it appears advisable to base the assay, not upon proteoses, as is done in the Pharmacopœia, but upon the simpler compounds beyond the proteose stage, the amino acids. These can be assayed by Sørenson's Formol Titration Method (6).

The assay methods for trypsin found in pharmaceutical literature are varied and difficult to interpret. For example, the United States Pharmacopœia states that pancreatin will digest 25 times its own weight of casein into proteoses. *New and Nonofficial Remedies* (1941) states that one part of Trypsin-Armour "digests at least 100 parts of casein according to the Fuld–Gross method." Trypsin–Fairchild, according to the same authority, when treated by the Fuld-Gross method will convert "200 times its weight of casein to the standard end-point." From these statements one may be led to believe that the latter preparation is 8 times as potent as Pancreatin U. S. P. even though the assertion is not directly made. When these preparations are assayed by identical methods, as by the Formol Titration Method, a true comparison can be made, and it will be found that the potencies of these preparations do not vary in the proportion implied.

The assay method of trypsin given in the United States Pharmacopœia under pancreatin is based upon the digestion of casein by proteolytic enzymes for a definite period of time, to the stage where the digestion mixture fails to give a precipitate when treated with a specially prepared mixture of acetic acid and alcohol. The acetic acid mixture

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is made by mixing 1 cc. of glacial acetic acid with 9 cc. of distilled water and 10 cc. of alcohol. The main objection to this method is the difficulty of determining the official end point, that is, the absence of any precipitate after the addition of three drops of the acetic acid mixture at the end of the one-hour digestion period.

Willson (5) states that "the end point is far from easy to determine with certainty," and that "inconsistent results are obtained by different workers." This variation seems to be caused, for the most part, by the difficulty of making a clear casein solution by the official method.

EXPERIMENTAL

The digestion period as given in the Pharmacopœia appears to be too long. The data given in Table I show that the trypsin in Pancreatin U. S. P. is about three times as potent as the official definition indicates. The Pharmacopœia states that no precipitate is obtained in one hour. Experimental evidence shows that a faint precipitate is obtained in twenty minutes when Pancreatin U. S. P. is assayed by the U. S. P. method, and that no precipitate is obtained in any of the samples after thirty minutes. It appears that Pancreatin U. S. P. is actually from two to three times as potent as is required in the official monograph.

TABLE I.—LENGTH OF TIME OF THE DIGESTION PERIOD REQUIRED TO ASSAY THE POTENCY OF PANCREATIN FOR TRYPSIN BY THE U. S. P. METHOD

Test Tube No.	Casein Sol., Cc.	Pancreatin Sol., Cc.	Water, Cc.	Time in Minutes	Acetic Acid Mixture
1	5	2	3	20	Faint ppt.
2	5	2	3	25	Very faint ppt.
3	5	2	3	25	No ppt.
4	5	2	3	25	Very faint ppt.
5	5	2	3	30	No ppt.
6	5	2	3	30	No ppt.
7	5	2	3	35	No ppt.
8	5	2	3	40	No ppt.
9	5	Blank	5	30	Heavy ppt.

A Comparison of Pancreatin U. S. P. with That of a "Triple Strength" Pancreatin.—An experiment was made in an attempt to compare the strength of Pancreatin U. S. P. with that of a "Triple Strength" Pancreatin. The U. S. P. method was followed, and the results show that neither gave a precipitate at the end of the one-hour digestion period. Each sample was then used in solutions of one-half the strength of the U. S. P. solution. The result was the same. Finally, solutions of one-third the strength of the U. S. P. solution were used, and in each case only a very faint precipitate was formed at the end of an hour. No difference could be distinguished in the samples as may be seen by a comparison of data given in Table II.

TABLE II.—COMPARISON OF PANCREATIN U. S. P. WITH A "TRIPLE STRENGTH" PANCREATIN BOTH ASSAYED BY THE U. S. P. METHOD

Test Tube No.	Pancreatin U. S. P. Strength of Pancreatin Solution Used	Effect of the Acetic Acid Mixture
1	Full strength	No ppt.
2	One-half strength	No ppt.
3	One-third strength	Faint ppt.
	Pancreatin "Triple Strength"	
1	Full strength	No ppt.
2	One-half strength	No ppt
3	One-third strength	Faint ppt.

Willson's Assay Method for Pancreatic Trypsin

Because of the difficulty of making a perfectly clear solution of casein wherein it would be easy to see the end point by precipitation, an entirely different type of assay was tried, an assay based upon the formation and determination of amino acids. This assay, based upon a modification of Sørenson's Formol Titration Method (6), improved upon by E. R. Smith in 1912 (7), and again by F. E. Willson in 1930 (8), is performed as follows:

Solutions Required.—No. 1: A 37% formaldehyde solution to which 1 cc. of 0.5% phenolphthalein in 50% ethyl alcohol is added. This is brought to neutrality with normal sodium hydroxide.

No. 2: A 4% solution of casein neutralized by normal sodium hydroxide of an amount determined by titrating a small amount of the casein solution in the presence of phenolphthalein.

No. 3: A 1% unfiltered pancreatin suspension in distilled water.

Procedure.—Heat the casein solution and, at the same time, a supply of distilled water to 55°. Add 24 cc. of this water to 25 cc. of the casein solution and place the mixture in the water bath at 55°. Add 1 cc. of the pancreatin solution to the first solution and allow it to digest for twenty minutes. While the solution is digesting withdraw 20 cc. from the blank, add 10 cc. of solution No. 1, and titrate to a pink color with 0.1 *N* sodium hydroxide. Withdraw 20 cc. of the digested solution into a 50-cc. Nessler tube, add 10 cc. of solution No. 1, mix well, and titrate with 0.1 *N* sodium hydroxide to the pink color of the blank.

As may be seen, since Willson's blank does not contain boiled pancreatin, it does not give the true picture that a control should give; for it does not allow for the possibility that the pancreatin may contain substances—acid in reaction—that would be titrated along with the decomposition products of casein in the final titration with 0.1 *N* sodium hydroxide. Furthermore, the period of time allowed

by Willson does not afford sufficient time to carry out certain controls that appear to be necessary.

An experiment was performed using time intervals of 20, 40, 60, and 80 minutes. When these results are represented graphically, it is seen that more than half the digestion takes place in the first twenty minutes. After the first twenty minutes the relationship of digestion to time is fairly constant. Since about 75% of the digestion takes place in the first forty minutes, and since this time was found to be a satisfactory working period, forty minutes was used for the digestion period of this enzyme in the modification which was adopted. The data obtained from this portion of the investigation are shown in Fig. 1 and Table III.

TABLE III.—RELATIONSHIP OF DIGESTION TO TIME IN EACH CASE 20 CC. OF THE DIGESTION MIXTURE ADDED TO 10 CC. OF NEUTRAL FORMALDEHYDE SOLUTION

Test Tube No.	Pancreatin "Triple Strength" Cc. of 0.1 *N* NaOH at Time Intervals of: 20 Min.	40 Min.	60 Min.	80 Min.
1. Digestion Mixture	5.35	6.35	6.95	7.50
2. Control 1 Willson	1.90	1.90	1.90	1.90
3. Control 2 (Containing Pancreatin Previously Boiled)	2.95	3.05	3.20	3.30

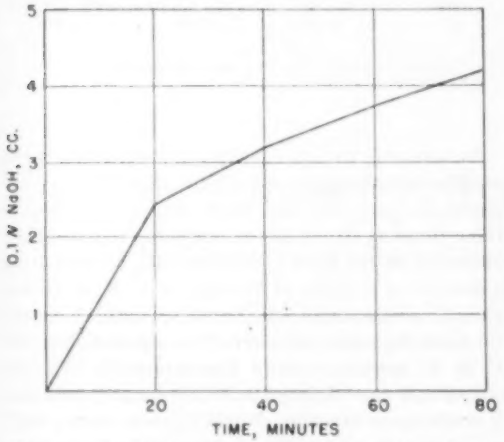

Fig. 1.—Graph showing relationship of digestion to time in assay of pancreatic trypsin.

Furthermore, it was found that when 5 cc. of the pancreatin suspension was used in place of the 1 cc., as used by Willson (8), there was more 0.1 *N* sodium hydroxide used, with the result that the larger figures cut down the percentage of error in the burette readings.

Another improvement in the method of assay was obtained by adding the digestion mixture to the formaldehyde solution already in the Nessler tube in order to stop the action of trypsin immediately at the end of the digestion period. This method reduces the possibility of error from the over-running of the time limit.

These data clearly indicate the value of using a control that contains the same amount of pancreatin, previously boiled, as is used in the digestion mixture.

Modification of Willson's Method.—Heat the casein solution and, at the same time, a supply of distilled water to 55°. Add 20 cc. of the water to 25 cc. of the casein solution and place in a water bath at 55°. Add 5 cc. of 1% pancreatin suspension and allow to digest for forty minutes. This is called the digestion mixture.

Control.—To 20 cc. of water add 25 cc. of the casein solution and place this mixture in the water bath at 55°. Then add 5 cc. of a 1% pancreatin suspension, which has been previously boiled for two minutes, and allow this mixture to stand for forty minutes.

Now add 10 cc. of the neutralized formaldehyde solution to each of two 50-cc. Nessler tubes. At the close of the digestion period withdraw 20 cc. from the digestion mixture, and from the control, and add to the formaldehyde solution in the Nessler tubes. Mix well and titrate each to a uniform pink color with 0.1 *N* sodium hydroxide.

The modifications of Willson's method may be briefly summarized as follows:

1. A control is used, which is identical to the digestion solution in every respect but one. The pancreatin solution has been boiled for two minutes before adding it to the mixture in order to destroy its enzymatic action.
2. The digestion period is forty minutes instead of twenty.
3. Five cubic centimeters of the 1% pancreatin suspension is used instead of 1 cc.
4. The formaldehyde solution is placed in a Nessler tube in advance and the digested mixture is added to it.

The Preparation of a Suspension of Casein More Nearly Homogeneous than Either Willson's or the Official Mixture.—Willson encountered some difficulty in dissolving the casein. In his directions for preparing a liter of solution he says: "40 Gm. of casein is added to 900 cc. of recently boiled distilled water to which approximately 25 cc. of 1 *N* NaOH has been added. The casein is brought into solution by stirring and mixing from time to time. If at the end of an hour or two all the casein is not dissolved a few more cc. of 1 *N* alkali are added to accomplish this."

The so-called solution obtained by this procedure was not homogeneous. Even upon adding more sodium hydroxide, and after allowing a longer period of time, the appearance did not improve. The casein seemed to swell; and after the suspension had been set aside, small undissolved particles settled to the bottom of the container.

In order to dissolve all the casein and to have a more nearly homogeneous suspension, the casein was

first powdered and sifted through a number 100 mesh sieve before it was treated with the solvent. When this powder was used a good suspension was formed, which was used with consistently satisfactory results. Pancreatin U. S. P. was assayed for casein digestive power with four different samples of casein by this modified method with nearly identical results. This indicates that the commercial samples of casein are similar in character to one another, and that they will give nearly homogeneous suspensions if the casein is first powdered and passed through a number 100 mesh sieve. All the experimental work, however, which followed, was done upon the same sample of casein.

Since it is impossible to put casein into solution by the U. S. P. method or by the Willson method, it appears advisable to modify the accepted procedure in order to bring about a more nearly homogeneous suspension of this substrate.

The Tryptic Activity of Various Samples of Commercial Pancreatin.—The discovery of pancreatic fibrosis and the renewed interest in pancreatic therapy for this condition, i.e., the inability to secrete trypsin, make it very important to know the tryptic activity of the available preparations of pancreatin or of the trypsin in order properly to estimate their medicinal value.

Commercial samples of pancreatin were assayed, in order to determine the exact amount of tryptic activity in them, by the modified Willson method instead of by the official assay for the minimum requirements indicated in the U. S. P. definition of pancreatin. If a sample is called "Triple Strength" Pancreatin, does it mean that it has three times the strength of the minimum requirements, or does it mean that it is three times as potent as ordinary Pancreatin U. S. P.? The U. S. P. test fails to distinguish between a sample which meets the minimum requirements and one which may be many times as potent.

TABLE IV.—COMMERICAL SAMPLES OF PANCREATIN ASSAYED BY THE MODIFIED WILLSON METHOD

Co.	Sample No.	Age in Mo.	Cc. of 0.1 *N* NaOH Used in Assay	Cc. of 0.1 *N* NaOH Used in Blank	Cc. of 0.1 *N* NaOH Actually Used
A	20	26	9.01	3.47	5.54
A	1A	24	9.55	2.97	6.58
A	11A	11	9.50	2.90	6.60
B	17	18	7.43	2.43	5.00
B	2A	67	7.03	2.28	4.75
B	3	67	7.18	2.57	4.61
B	3A	103	6.39	2.77	3.62
B	8A	67	6.98	2.72	4.26
C	10A	35	7.57	2.52	5.05
C	4A	53	7.47	2.47	5.00
C	4	89	7.77	2.57	5.20
C	12	23	7.60	2.68	4.92
C	3X[a]	Age unknown	10.36	3.28	7.08
D	7A	112	7.77	2.52	5.25
D	14	100	7.03	2.52	4.51
D	3Y[b]	Age unknown	10.58	3.37	7.21

[a] "Triple Strength" Pancreatin.
[b] "Triple Strength" Enteric Coated Capsule.

TABLE V.—SAMPLES OF TRYPSIN OBTAINED FROM VARIOUS SOURCES ASSAYED BY THE MODIFIED WILLSON METHOD

Sample No.	Cc. of 0.1 *N* NaOH Used in Assay	Cc. of 0.1 *N* NaOH Used in Blank	Cc. of 0.1 *N* NaOH Actually Used
1	9.06	3.14	5.02
1A	8.91	3.15	5.76
2	9.30	3.00	6.30
3[a]	10.25	3.46	6.79
3A[a]	10.30	3.59	6.71
4[b]	9.45	3.15	6.30

[a] "Triple Strength" Enteric Coated Tablets.
[b] Reference Standard Trypsin.

By assaying by the modified Willson method the samples of pancreatin obtained from different companies, it was found that most samples were two to three times as strong as the minimum requirements indicated on the label. Furthermore, in comparing a number of samples of Pancreatin U. S. P. (Company C) with a sample of "Triple Strength" Pancreatin from the same company, it was found that the U. S. P. samples were of approximately 70% the strength of the "Triple Strength" sample; whereas it would seem that they should be more nearly 33% as strong as the "Triple Strength" Pancreatin. However, a noticeable difference in the samples can be determined by the modified Willson method, whereas a previous experiment, when Pancreatin U. S. P. and "Triple Strength" Pancreatin were assayed by the U. S. P. method, merely showed that they both met the U. S. P. minimum requirements.

SUMMARY

1. The trypsin designated in the U. S. P. Pancreatin is a mixture of proteolytic enzymes.

2. The official assay is unsatisfactory, as no definite standard, with which different samples can be compared, is set up.

3. A modification of Willson's assay of pancreatin has been undertaken and has worked out successfully.

4. A number of commercial samples of pancreatin have been obtained and assayed by this method. A comparison of the strength of trypsin in various samples of pancreatin can be expressed in terms of the number of cc. of 0.1 *N* sodium hydroxide used to neutralize the amino acids produced

by digestion upon a specially prepared solution of casein.

5. A modification of both the U. S. P. and the Willson methods has been made in the preparation of the casein solution.

6. Samples of commercial trypsin were also assayed by the modified Willson method, and the results have been compared with U. S. Pharmacopœia Reference Trypsin.

7. It has been demonstrated by these assays that "Triple Strength" Pancreatin possesses three times the potency of the minimum requirements as stated in the official monograph; but, that it is misleading to imply that it posesses three times the potency of Pancreatin U. S. P.

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The Carboxypeptidase Activity of Pancreatin U. S. P.*

By CHARLES W. BAUER† and ALPHONSE M. MISKINIS‡

Pancreatin U. S. P. contains the enzyme carboxypeptidase. It is a relatively stable enzyme. Pumpkin seed globulin serves as an excellent substrate for its analysis. Its activity may be expressed in terms of cc. of 0.02 *N* sodium hydroxide required to neutralize the amino acids produced from a definite amount of pumpkin seed *alpha*-globulin.

UNTIL a few years ago it was thought that proteins were first digested to peptones by the pepsin in the stomach, and were then completely digested into absorbable units by the trypsin of the pancreas. It is now known that the enzyme trypsin, in the strict meaning of the term, does not complete the digestion of proteins into absorbable units. It has been found that the enzymes aminopeptidase and carboxypeptidase accomplish this result. Since carboxypeptidase is secreted by the pancreas, it is of interest to determine whether this enzyme is present in the official preparation of pancreatin.

The object of this investigation was to determine the presence and the relative amounts of carboxypeptidase in different samples of Pancreatin U. S. P.

In 1927 Waldschmidt-Leitz (1) and coworkers discovered the enzyme carboxypolypeptidase as the zymogen in the pancreas of certain of the higher animals. Two years later Waldschmidt-Leitz and Purr (2) shortened the name of carboxypolypeptidase to carboxypeptidase.

According to Bergmann (3) carboxypeptidase splits an end amino acid from its substrate, the enzyme attacking only those substrates containing a free carboxyl group next to the peptide linkage. The work of Bergmann and Fruton (4) indicates that the CO—NH—CH(R)—COOH groups are arranged in a counterclockwise order in which the alpha hydrogen atom of the CH(R) group is directed toward the enzyme. Anson (5) found carboxypeptidase to be the only known proteolytic enzyme that acts in the presence of formaldehyde.

One of the synthetic substrates employed for the measurement of carboxypeptidase activity is the expensive chloracetyl-*l*-tyrosine which was used by Waldschmidt-Leitz (2). Anson (7) found that a peptic digest

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of edestin was a satisfactory substrate for his investigations of carboxypeptidase.

Waldschmidt-Leitz (1) asserts that the zymogen found in the pancreas is activated by the enterokinase of the intestines. It has been suggested by Sumner and Somers (6) that the activation of the zymogen may be due to the action of trypsin.

MATERIALS AND METHODS

Substitute Substrate for Edestin.—Since chloracetyl-*l*-tyrosine was too expensive to be used in this investigation, the peptic digest of edestin was first employed as the substrate. The formalized solution of edestin was prepared according to Anson (7), using edestin (Eimer and Amend), decolorizing charcoal (Merck), granulated pepsin (Wilson Laboratories), and Filter-Cel (Johns-Manville). After several preliminary experiments with the edestin solution, the supply of edestin ran out, and no more could be obtained anywhere. The experiments performed with edestin clearly indicated the presence of carboxypeptidase in Pancreatin U. S. P. In order to continue this investigation it became necessary to find a satisfactory substrate to take the place of edestin.

In 1940 Vickery, Smith, and Nolan (8) reported the use of pumpkin seed globulin as a substitute for the globulin edestin obtained from hemp seed. These workers found the yield of globulin prepared from pumpkin seed by their method to be about 10%, by weight of the seed, a percentage somewhat higher than that of hemp seed. An added advantage of pumpkin seed globulin is that it is cheaper than edestin. Hence, pumpkin seed globulin was used as the substrate for the major portion of the experimental work reported in this paper.

Theory of Formol Titration and Modification.—In the determination of proteolytic enzymes other than carboxypeptidase by formol titration, the usual procedure for obtaining a standard consists of adding formaldehyde to the substrate solution to stop the enzymatic digestion, then of adding phenolphthalein and a sufficient amount of sodium hydroxide to bring the solution to a standard color. This preparation constitutes the standard or control. The increase in amount of alkali used over that of the control is a measure of the number of amide linkages split. This procedure is inadequate, according to Anson (7), and has to be slightly modified for the determination of carboxypeptidase, since the carboxypeptidase activity, unlike other proteolytic activities, is not stopped by the addition of formaldehyde. The blank is run on a substrate solution in the absence of the enzymatic preparation but in the presence of formaldehyde, phenolphthalein, and sodium hydroxide.

In the experimental investigation which follows, the blank was further modified by adding a boiled enzyme preparation which contained some amino acids not obtained from carboxypeptidase digestion. This modification eliminates the danger of including the amino acids, which are normally present in the crude pancreatin, from being considered to be the result of enzymatic action upon the substrate.

Preparation of Pancreatin Suspension.—A 5% pancreatin suspension was found by trial to be very satisfactory for this investigation. The suspension of pancreatin should be as homogeneous as possible in order that equal measurements may contain about equal amounts of pancreatin. All suspensions were made in distilled water about one half-hour before starting the assays.

Since heating might possibly have hydrolyzed some additional acids in the pancreatin, it was necessary to determine the acidity of both the boiled and the unboiled pancreatin suspensions. The effect of heat on the hydrolysis of pancreatic proteins had to be determined in order to have a correct control or blank. Phenolphthalein was used as the indicator, and the boiled suspension was first cooled to room temperature before assaying. The experimental results indicate that boiling has no effect upon the acidity of the preparation, as is shown by the fact that the amount of alkali was practically the same in each instance.

While the acidity of different samples of pancreatin may vary, the difference between the boiled and unboiled pancreatin in any one sample is about the same.

Preparation of Standard Formaldehyde Solution.—To 50 cc. of water add 15 cc. of formaldehyde 37%, 2 cc. of 0.5% phenolphthalein indicator solution, enough 5 *N* sodium hydroxide to make the solution just detectably pink, and enough distilled water to make the total volume measure 100 cc. This solution contains the same amount of formaldehyde as the formalized substrate solution.

Preparation of Pumpkin Seed Globulin.—To 150 Gm. of freshly ground pumpkin seed, in a previously warmed dry mortar, add sufficient 10% sodium chloride solution, warmed to 60–65°, to form a very soft paste. Triturate; add about one liter of sodium chloride solution and triturate for an additional five minutes. Strain through a number 20 sieve into a large evaporating dish and return the residue to the mortar for a second treatment. Use the remainder of the sodium chloride solution, two liters of which are prepared for the preparation, for the second extraction. Triturate again; then strain the contents into the large evaporating dish. Heat for one hour at 60–65° with constant stirring. Transfer the contents to a two-liter flask and tie a double fold of cheesecloth over the neck of the flask in the form of a small sack.

Invert the flask and contents into a stepfolded filter paper that has been washed with warm 10% solution of sodium chloride. It is desirable to use a water-jacketed funnel, maintained at 60–65°, for the filtration process, for, if the funnel and paper become cold, the globulin will precipitate and will clog the pores of the filter paper.

Dilute the filtrate with distilled water to a 2% concentration of sodium chloride and set it aside at room temperature for four to five hours; then place it in the refrigerator at 5° for twelve hours. Then siphon off the supernatant liquid. Filter the precipitate through a large Jena filter or a glass sintered filter, and wash the precipitate twice with a small amount of distilled water. Dry the precipitate over a steam-heated radiator or in a warm place, powder it finely, and pass it through a bolting cloth. The extracted globulin is light yellow-brown in color and behaves very similarly to edestin, as a control experiment showed. The yield by the foregoing method is about 6–7%.

The pumpkin seed globulin served as a satisfactory substrate for the determination of carboxypeptidase activity in Pancreatin U. S. P. Anson's (7) procedure was used to prepare the formalized substrate solution, but pumpkin seed globulin was substituted for the edestin.

Preparation of Digestion and Control Flasks and the Method of Assay

The Preparation of the Standard Color for the Blank.—To flask 1S, the standard color flask, add 10 cc. of 5% boiled pancreatin suspension previously cooled to room temperature, one drop of phenolphthalein indicator, and titrate to a pink color with 0.02 *N* sodium hydroxide in order to neutralize the amino acids present in the pancreatin suspension. Then add 5 cc. of neutralized standard formaldehyde solution. The pink color disappears on the addition of the formaldehyde solution because of the release of the carboxyl groups of the amino acids present as zwitter ions in the neutralized pancreatin. Make the suspension pink again with 0.02 *N* sodium hydroxide. Stopper the flask, and let this preparation represent the color standard for flasks 2a and 2b.

Blank Determination of Flasks 2a and 2b.—Add 10 cc. of 5% cooled, boiled pancreatin suspension, and one drop of phenolphthalein indicator to flasks 2a and 2b, which are to be employed as blanks for the digestion flasks 4a and 4b. Titrate the mixtures with 0.02 *N* sodium hydroxide to the same pink color as that of the standard color flask, 1S; then add 5 cc. of formalized substrate solution. The pink color disappears after this addition, but returns on the addition of more alkali. After the desired pink color is obtained, stopper the flasks and place them in a constant temperature bath for thirty minutes at 37°. The standard color flask, 1S is also placed in the constant temperature bath at the same time. The amounts of 0.02 *N* sodium hydroxide used need not be recorded, for the alkali is used only to neutralize the acids in the pancreatin suspension as well as any amino acids that may be liberated when the formaldehyde solution is added. None of these acids is a product of the digestion with the enzyme preparation.

At the end of thirty minutes—the period allowed for digestion in flasks 4a and 4b—remove the flasks from the temperature bath. Titrate the contents of the flasks 2a and 2b with 0.02 *N* sodium hydroxide until the color of each matches that of the standard flask. Record the number of cc. of 0.02 *N* sodium hydroxide required to produce the desired color in flasks 2a and 2b. This is the amount, in terms of cc. of 0.02 *N* sodium hydroxide, necessary to neutralize the acids that may be produced during the thirty-minute digestion period. Theoretically there should be no amino acids produced, since the pancreatin suspension was boiled for seventy-five seconds to destroy all enzymatic activity. Flasks 2a and 2b are employed as blanks for the digestion flasks 4a and 4b, which contain the unboiled, enzymatically active, pancreatin suspension.

The Preparation of the Standard Color for the Digestion Flasks.—To flask 3S, which is to be the standard color flask for flasks 4a and 4b, in which digestion occurs, add 10 cc. of unboiled 5 per cent pancreatin suspension, one drop of phenolphthalein indicator, and titrate to a pink color with 0.02 *N* sodium hydroxide. Add 5 cc. of neutral standard formaldehyde solution and again add 0.02 *N* sodium hydroxide until the standard color is obtained. Stopper the flask, and use it as the standard color flask for 4a and 4b.

The Preparation of the Flasks 4a and 4b.—Add 10 cc. of unboiled 5% pancreatin suspension to flasks 4a and 4b; then add one drop of phenolphthalein indicator and titrate to the standard color. Then add 5 cc. of neutral formalized substrate solution and immediately titrate to the standard color; stopper, and place the three flasks in the constant temperature bath at 37° for thirty minutes. The amount of sodium hydroxide is not recorded. However, after the removal of the flasks from the temperature bath, the number of cc. of 0.02 *N* sodium hydroxide required is an indication of the degree of carboxypeptidase activity in the pancreatin suspension.

At the end of thirty minutes remove the flasks from the thermostatic bath. If the color of the standard color flask, 3S, be markedly faded or be of such a weak shade that it is difficult to compare or reproduce, introduce a known amount of 0.02 *N* sodium hydroxide until the desired color is obtained. Titrate the other flasks, 4a and 4b, with 0.02 *N* sodium hydroxide until their color matches that of the standard (3S). Subtract the number of cc. of 0.02 *N* sodium hydroxide used to produce the desired color in the standard color flasks, 3S, from the total number of cc. used in each of the two digestion flasks 4a and 4b.

Interpretation of the Assay Described.—Boiling the pancreatin suspension for seventy-five seconds destroys the enzyme present in the blank flasks 2a and 2b. Therefore, if the average number of cc. of 0.02 *N* sodium hydroxide used in flasks 2a and 2b equals the average number used in 4a and 4b, which contain the unboiled pancreatin suspension, the activity shown would not be caused by the enzyme, but by something else. However, if the average amount of 0.02 *N* sodium hydroxide used in 2a

and 2b be less than the average amount used in 4a and 4b, it may be assumed that part or all of the activity was caused by the carboxypeptidase, because carboxypeptidase is the only known proteolytic enzyme that will act in the presence of formaldehyde.

Experimental Data.—A sample of Pancreatin U. S. P. was obtained from one of the larger pharmaceutical houses. The determination of carboxypeptidase was determined by the previously described method of assay. The average requirement of the control flasks was 0.7 cc. of 0.02 *N* sodium hydroxide while the average requirement of the digestion flasks was 5.9 cc. The control average, 0.7 cc., subtracted from the digestion flask average, 5.9 cc., gives 5.2 cc. of 0.02 *N* sodium hydroxide used. This is the actual carboxypeptidase activity measured in terms of cc. of 0.02 *N* sodium hydroxide.

TABLE I.—THE AMOUNT OF CARBOXYPEPTIDASE IN SAMPLES OF PANCREATIN OBTAINED FROM COMPANIES A, B, C, AND D EXPRESSED IN TERMS OF CC. OF 0.02 *N* SODIUM HYDROXIDE REQUIRED TO NEUTRALIZE THE ACIDS LIBERATED BY THE ENZYME

Co.	Sample No.	Age in Mo.	Av. No. of Cc. of 0.02 *N* NaOH for Blanks	Av. No. of Cc. of 0.02 *N* NaOH for Digestion Flasks	Av. Carboxypeptidase Activity in Terms of Cc. of 0.02 *N* NaOH
A	1A	24	0.99	5.275	4.285
A	11A	11	1.15	5.190	4.040
B	4A	53	0.775	5.300	4.525
B	10A	35	0.675	5.275	4.600
B	12	23	0.500	5.400	4.900
B	12A	21	0.600	5.120	4.520
C	7	66	0.685	5.335	4.650
C	7A	112	0.900	5.350	4.450
C	14	100	0.400	5.700	5.300
C	15	46	1.115	6.100	4.985
D	3	67	0.700	5.650	4.950
D	2A	67	0.700	5.900	5.200
D	3A	103	0.635	6.100	5.465
D	17	18	0.800	6.150	5.350
D	5	73	0.410	5.950	5.540
D	8A	67	0.475	6.010	5.535
D	6	163	0.690	5.650	4.960
D	6A	85	1.060	5.500	4.440

The most recently manufactured sample of pancreatin assayed was eleven months old, the oldest sample was 163 months old, while the average age of the samples was about 53 months. The average carboxypeptidase activity of 10 cc. of a 5% suspension of pancreatin on 5 cc. of a pumpkin seed globulin substrate made according to the Anson method, expressed in terms of 0.02 *N* sodium hydroxide solution is 4.71 cc.

CONCLUSION

The experimental data definitely indicate the presence of carboxypeptidase in Pancreatin U. S. P. Since Pancreatin U. S. P. contains trypsin, a carboxypeptidase activator, it was impossible to determine the amount of enzyme in the form of its zymogen. The assay is based entirely upon active carboxypeptidase. Since all experimental work was carried out on an impure enzymatic preparation, it was impossible to express the amount of enzyme in Anson units (7), which are based on a crystalline carboxypeptidase. However, the amount of carboxypeptidase may be expressed in terms of the cc. of 0.02 *N* sodium hydroxide required to neutralize the amino acids liberated by carboxypeptidase from a definite amount of substrate. The amount of carboxypeptidase in samples of Pancreatin U. S. P. seems not to be affected to any significant extent by the age of the samples.

SUMMARY

1. Pancreatin U. S. P. contains carboxypeptidase.

2. Pancreatin suspensions may be boiled to destroy the enzyme without affecting the degree of acidity.

3. Pumpkin seed globulin is a satisfactory substrate for the determination of carboxypeptidase.

4. Boiled Pancreatin must be used in the control in order to avoid a misinterpretation of the carboxypeptidase activity of the unboiled pancreatin on the pumpkin seed globulin.

5. The activity of carboxypeptidase may be expressed in terms of cc. of 0.02 *N* NaOH required to neutralize the amino acids produced from a definite amount of substrate.

6. The amount of carboxypeptidase in samples of Pancreatin U. S. P. was practically the same for all samples, which were obtained from different sources, and which were stored for different periods of time. This uniformity seems to indicate that carboxypeptidase is a fairly stable enzyme.

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Chlorosulfonic Acid in the Synthesis of DDT and Its *p*-Halogen Analogues*

By W. T. SUMERFORD†

The chlorosulfonic acid method of preparing DDT is outlined, its general application discussed, and the compounds which were prepared are described.

SINCE the discovery of the insecticidal activity of DDT [1-trichloro-2,2-*bis*(*p*-chlorophenyl)ethane] by Müller (1) in 1939, the chemistry of this and related compounds has been developed in this country and elsewhere at a very rapid rate. Among the first analogues of DDT to suggest themselves as potentially superior substitutes were its *p*-halogen analogues: (*a*) 1-trichloro-2,2-*bis*(*p*-fluorophenyl)ethane (DFDT), (*b*) 1-trichloro-2,2-*bis*(*p*-bromophenyl)ethane (DBrDT), and (*c*) 1-trichloro-2,2-*bis*-(*p*-iodophenyl)ethane (DIDT).

Baeyer was the first to condense two moles of benzene with one mole of chloral to give 1-trichloro-2,2-*bis*(phenyl)ethane (2) herein referred to as (DPE).

Both DDT and its *p*-bromine analogue (DBrDT) were prepared and described by Zeidler in the first report on compounds of the DDT type (3).

The *p*-fluorine analogue of DDT (DFDT) was introduced in Germany during World War II under the names of "Fluorogesarol" (GIX) (4). It is said to be seven times as effective against flies and to cost ten times as much as DDT (5). DFDT was prepared by condensing two moles of fluorobenzene and one mole of chloral using sulfuric acid (4), or by condensing two moles of fluorobenzene and one mole chloral acetal in the presence of chlorosulfonic acid (6). It has been described as a liquid, b. p. 160–180° at 8 mm. (7) and as a solid, m. p. 31° (8). No analyses for this compound were found.

It has been shown that iodobenzene readily condenses with chloral with the formation of 1-trichloro-2,2-*bis*(*p*-iodophenyl) ethane (DIDT), colorless prisms, m. p. 172° (9).

All of the above condensations, unless otherwise noted, were brought about by the use of concentrated sulfuric acid. The order of mixing the reactants and the acid, however, was not the same in each case.

EXPERIMENTAL

The use of chlorosulfonic acid in condensing two moles of chlorobenzene and one mole of chloral to yield DDT, first reported from this Laboratory (10), has been extended to the synthesis of 1-trichloro-2,2-*bis*(phenyl)ethane and its *p,p'*-dihalogen derivatives.

The procedure used to prepare and purify these derivatives is essentially the same as that previously described (10). One mole of chloral is mixed with 2 moles of benzene or the appropriate halogen derivative. This solution is cooled to 5° and to it is added 1.8 moles of chlorosulfonic acid in divided portions and under constant stirring. After stirring for three hours, the reaction mixture is poured into chipped ice and the crude product is collected on a Buchner funnel. It is boiled with ten times its weight of water and again collected after cooling.

The pertinent data for the compounds prepared by this method are given in Table I.

TABLE I

Compound	Recrystallizing Solvent	Yield, %	M. P., °C
DPE	MeOH—H_2O[b]	60	62
DFDT[a]	EtOH—H_2O[b]	68	40
DDT	Et_2O—MeOH (1–3)[c]	70	106
DBrDT	Et_2O—EtOH (2–1)	75	139
DIDT	Et_2O—EtOH (1–1)	80	172

[a] This compound was solidified prior to recrystallization by placing it in a chest with dry ice. Theory: C% 52.20 H% 2.80. Found: C, 51.72. H, 2.80.
[b] Dissolve in appropriate amounts of the respective alcohols, and add water to incipient precipitation.
[c] This compound may be purified also by washing it twice with an equal weight of methanol.

Biological Activity.—Determinations have been made of the relative toxicity of these compounds

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toward mosquito larvae, mosquito pupae, and two species of fish. The unsubstituted compound, DPE, was comparatively nontoxic. The median lethal doses of the *p,p'*-dihalogen derivatives were approximately 0.1 p. p. m. for goldfish, 0.01 p. p. m. for Gambusia, and 0.001 p. p. m. for larvae. The pupae were more resistant than the larvae or the Gambusia.

A detailed report on these tests will be published elsewhere.

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Book Reviews

Advancing Fronts in Chemistry. Volume II, Chemotherapy. Edited by WENDELL H. POWERS. Reinhold Publishing Corporation, 330 West 42nd St., New York, 1946. 156 pages. 14.5 x 23.0 cm. Price, $3.50.

In 1945 the Wayne University sponsored a course of academic studies entitled "Frontiers in Chemistry." It was certainly fitting that chemotherapy, a science which has given so much to the health of mankind, should fit into such a program. This book is a compilation of all of the lectures presented in the chemotherapeutic field with the exception of Dr. H. E. Carter's lecture on antibiotics. The following lectures were included:

"Chemotherapy in Experimental Tuberculosis," by William H. Feldman;
"Synthetic Antispasmodics," by Frederick F. Blicke;
"Chemistry of the Sulfa Drugs," by E. H. Northey;
"The Antimalarial Problem," by Harry S. Mosher;
"Organometallic Compounds as Chemotherapeutic Agents," by C. Kenneth Banks;
"Past Developments and Present Needs in the Chemotherapy of Parasitic Diseases," by Willard H. Wright.

In all cases, the emphasis has been placed on recent developments and no attempt was made to completely survey the literature in these specific fields. Enough historical material was presented to provide a satisfactory setting. Many of the authors were particularly stimulating by indicating probable future trends and future needs.

The inclusion of photographs of the authors and brief biographies will be a delight to many since they help create the illustion that the reader attended this auspicious lecture series.—MELVIN W. GREEN.

Chemistry of Food and Nutrition, Edition 7, by HENRY C. SHERMAN. The Macmillan Company, New York, 1946. viii + 675 pages. 13.5 x 21 cm. Price, $3.75.

Nutrition as a chemical science and Professor Sherman's excellent text cannot be separated without leaving a tremendous void. It is difficult to realize that the first *Chemistry of Food and Nutrition* became available in 1911. Many spectacular and dramatic events have taken place in the intervening years. Professor Sherman has not only been most faithful in recording these events, he has actively participated in most of them.

The seventh edition of this book, like the others represents a thorough revision where swift moving events demanded it. Because the author has taken pains to make clear the certainty degree of his "facts," the undergraduate is not at a loss to know the "truth."

The documentation is selective and exceptionally good.—MELVIN W. GREEN.

Qualitative Analyses by Spot Tests, by FRITZ FEIGL. Third English Edition, 1946. Elsevier Publishing Company, New York. xvi + 574 pages. 14.5 x 23 cm. Price, $8.00.

The third English edition of this standard work on spot testing is much like previous editions in form. It has been completely revised, however. Not only have new tests been added but the tests previously given have been critically evaluated. All manipulations previously described separately in *Laboratory Manual of Spot Tests* (Academic Press, 1943) have been included. Altogether the text contains 350 detailed and about 100 orienting tests in addition to about 175 suggested procedures. This edition was translated by Professor Ralph E. Oesper.—MELVIN W. GREEN.